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Extraction, Purification and Characterization of a Pure Peptide from Soybean to Demonstrate Anti-Proliferation Activity on Human Cancer Cells and Test the Ability of Soy Peptide Fractions in Reducing the Activity of Angiotensin-I Converting Enzyme

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Extraction, Purification and Characterization of a Pure Peptide from Soybean to Demonstrate
Anti-Proliferation Activity on Human Cancer Cells and Test the Ability of Soy Peptide Fractions
in Reducing the Activity of Angiotensin-I Converting Enzyme

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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Abstract

Proteins are of major interest for nutritional value, functionality and sensory characteristics in foods, and biological activity as health-promoting ingredients. A large number of potential biological activities are encrypted within the primary structures of animal and plant proteins. This research has been based on utilizing a legume by-product, soybean meal, to obtain value-added protein products. Forty four Arkansas grown soybean lines were subjected to analysis for protein and amino acid (profile) content to select lines that have high protein attributes. Three soybean lines, R95-1705 (high yielding, non-transgenic and high protein), N98-445A and S03-543CR (bred for high oleic acid - monounsaturated fatty acid) were selected for further study. Seeds from the three soybean lines were ground and defatted to prepare the meals. The protein isolates (SPI) with highest (90-93%) purity prepared from the three meals were hydrolyzed using a food grade enzyme, Alcalase 2.4L, under optimal hydrolysis conditions to prepare protein hydrolysates (pools of peptides). The protein hydrolysates were tested using simulated gastric and intestinal juices to determine the gastro-intestinal (GI) resistance of the peptides. The GI resistant hydrolysates were fractionated to collect distinct molecular size cut-offs of <5, 5-10, 10-50 and >50kDa using ultra-filtration technique. These peptide fractions were tested for biological activities including inhibition of ACE-I activity and cancer cell proliferation inhibition using specific assays. Research highlights include demonstration of ACE-I activity inhibition and cancer cell anti-proliferation activity by the GI-resistant peptides from the soybean lines in comparison to positive controls, Captopril (anti-hypertensive drug) and Genistein (anti-cancer agent). A comprehensive screening provided definitive selection criteria for choosing the 10-50kDa fraction from N98-445A for further purification to separate a single pure peptide with enhanced biological activity. A purified peptide with a sequence of 158 amino acids and

molecular weight of 18kDa from the 10-50kDa fraction showed nearly 75 and 80% inhibition of blood and colon cancer cells respectively. Hence, the importance of this investigation is in the potential ability of a peptides obtained from soybean meal protein to sustain a progressive impact on human health condition.

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Dedication

This Ph D. Dissertation is dedicated to my grandfather, late Mr. Ramakrishniah Rayaprolu.

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List of published papers

1. Srinivas Rayaprolu, Navam Hettiarachchy, Ronny Horax, Eswaranandam Satchithanandam, Pengyin Chen and Andy Mauromoustakos. 2015. Amino acid profiles of 44 soybean lines and ACE-I inhibitory activities of peptide fractions from selected lines. *Journal of the American Oil Chemists' Society*, 92: 1023-1033.
2. Srinivas J Rayaprolu, Navam S Hettiarachchy, Pengyin Chen, Arvind Kannan, and Andronikos Mauromostakos. 2013. Peptides Derived from High Oleic Acid Soybean Meals Inhibit Colon, Liver and Lung Cancer Cell Growth. *Journal of Food Research International*, 50(1): 282-288.

CHAPTER 1

Introduction

Soybean (*Glycine max*) is a leguminous plant grown as a pulse crop in various parts of the world. The pods contain seeds which are the edible parts of the plant and known for the rich lipid and protein content. United States is the largest producer and exporter of soy in the world. According to United States Department of Agriculture (USDA), soybeans account for about 90 percent of U.S. oilseed production. In 2014, the American Soybean Association puts the farm production value of soybean at \$40.4 billion (ASA, 2015), and U.S exports stand at 42% of total world trade in soybeans. The increase in soy production in the US is due to the high yield potential of the crop and the increase in demand for various soy products in the globally. Genetic engineering techniques are developed to obtain seeds with high protein, and oil content with increased economic output.

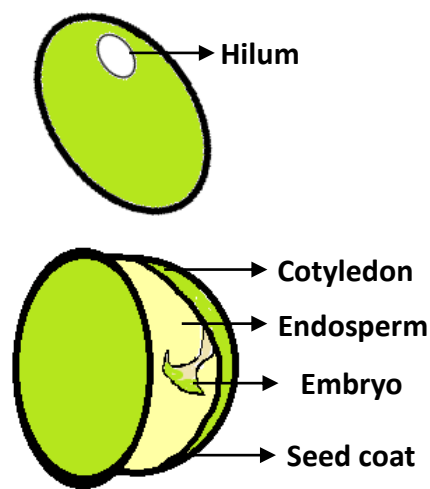


Figure 1.1. Illustration of soybean seed parts.

Soybeans seeds are small, round and green in color at maturity but turn yellow when they are dried for storage. The seed parts are illustrated in Figure 1.1: a hilum (small brown spot), seed coat (a thin layer outside that protects the seed), cotyledons (the part that stores food for the embryo), and the embryo (that develops into a new plant). The endosperm contains various components including carbohydrates, proteins, lipids, vitamins and minerals that are essential for germination of the embryo. Hence, the endosperm has commercial value as soybeans are chiefly grown for their oil which is used in various industries.

Soybean oil is widely used in the food industry and is made of five major fatty acids: palmitic, stearic, oleic, linoleic, and linolenic. Higher percent of linolenic acid causes spoilage,

and rancidity in foods, which is due to hydrogenation of linolenic acid, a common practice in the food industry employed to enhance the stability of the product. Hence, soybean breeders are developing cultivars that consistently produce oil with lesser amount of polyunsaturated linolenic acid, while increasing the percent of monounsaturated oleic acid.

Genotypic variations have been found to be the cause for differences, not only in the oil content, but also in the concentration of crude protein, possibly even in the amino acid make-up. There is an increasing demand for high oleic acid soybeans in the vegetable oil industry and the soy meal, which is the byproduct after processing of seeds, is a source of high quality protein. Methionine is an essential amino acid which is deficient in soybean. Hence, there is a possibility that high oleic acid soybean meal may be containing higher amounts of amino acids, methionine and cystine. Hence, we propose to look at the difference in the make-up of peptides between the high protein (Hutcheson, S97-1688, R95-1705), and the high oleic acid soybean (N98-4445A) varieties.

We know that proteins are made of amino acids and can be hydrolyzed at specific sites with controlled digestion to yield different sized peptides. Bioactive peptides are food derived that, in addition to the nutritional value exert a physiological effect in the body. Soybean protein has been identified as a valuable source of bioactive peptides which can be incorporated into foods. Preparation of peptides and their fractionation for testing their activity against disease cells has been successfully studied by several researchers. Studies have shown that exposure to the digestive enzymes in the stomach has an influence on the bioavailability of the peptides in controlling the diseases (Park et al 2007, Miguel et al 2006). Since different sized fragments of peptides would be generated by the enzymatic hydrolysis, fractionation of the GI resistant peptides hydrolysates with definite molecular weight cut-off membranes needs investigation.

The delivery of these peptides to the target organs is important for maximum availability of the compound to exert its bioactivity, and controlling the spread of the disease. In vivo studies have been conducted to test micro-encapsulation of the peptides and their bioavailability depending on prolonged duration. There was a significant protective effect of encapsulation against the gastric and intestinal juices and has shown a correlation between the peptide availability and the control of the disease (Chen et al 2003). To assess the delivery system, micro-encapsulation of these bioactive peptides using nanotechnology for food product development and applications have been investigated (Alessa *et al.*, 2014). The shelf-life and sensory quality of the peptides in the food products have been tested by researchers for developing food products (Khairallah, 2011, Alessa *et al.*, 2014). There is increasing interest over incorporation of bioactive peptides into foods in the United States. Individuals are supplementing traditional health care products by switching to nutraceuticals and functional foods.

Bioactive substances of food origin can be defined as components (genuine or generated) of consumption ready food which may exert regulative activities in the human organism beyond basic nutrition. A great diversity of food-derived bioactive substances having a non-nutrient character can be considered as an aid in maintaining good health. Biologically active proteins and peptides are becoming prevalent candidates for customized and unconventional treatment strategies in disease conditions where conventional therapeutics have been proven to be burdensome or expensive. Additionally, food products incorporated with bioactive peptides are limited and still evolving in the United States, where there is an ever increasing need for these products to reduce the risk of chronic diseases. Legumes like soybeans have gained much prominence in the past few decades for their high protein value, increasing production, and

sustained use in comparison to other food products. Research on bioactive peptides from various sources, their generation and functional aspects have intensified over the past two decades. Pilot-scale production of peptides is limited by the lack of suitable cost-effective technologies. Several of the biological properties of legumes are attributed to physiologically active peptides in these proteins (Korhonen and Pihlanto, 2003; Chiang et al., 2006; De Mejia and Ben, 2006). The amino acid sequences of these peptides dictate their activities in vivo affecting cardiovascular or endocrine or immune or nervous systems. Some of the bioactive proteins and peptides have shown immunomodulating, antihypertensive, osteoprotective, antilipemic, opiate, antioxidative and antimicrobial properties (Moller et al., 2008).

Heart disease and cancer are the leading causes of death in the United States. Hence, research project designed to prepare and identify soy peptides for bioactive properties against hypertension, a chief risk factor for cardiovascular disease (CVD), as well as various kinds of cancers are essential. Sources like milk, other legumes and cereal grains have the potential to provide biologically active peptides. However, factors like abundant availability, protein yield and cost may promote soybean meal for consideration as a good source of peptides in comparison to others. Soybean meal, a co-/by-product of soybean oil industry, is a good source of protein (approximately 45-50%) and can be a good starting material for creating new bioactive peptides and posing no difficulties during extraction.

The primary objective of this research was to prepare protein hydrolysates and peptides from soy protein isolate which was extracted from soybean meal by protease digestion at optimum degree of hydrolysis. In order for the peptides to impart their bioactivities, they need to be resistant to the enzymatic gastro intestinal (GI) environment (Park et al 2007). Although several broad studies confirmed bioactive peptides, assessment of gastrointestinal resistance due

to enzymes have not been prominently assessed. Hence, gastrointestinal environment resistant peptides were produced and tested for bioactivity. Consequently, the hydrolysates were fractionated based on molecular size cut-offs before testing the activities against ACE-I and cancer cell proliferation. This project provides basis for developing GI juice resistant bioactive peptides against different ACE-I activity (potential anti-hypertension) and cancers. This was followed by enhanced bioactivity through purification of the peptide pool to isolate single pure peptide. Full characterization of single pure peptide(s) with highest activity was conducted using liquid chromatography coupled with mass spectrometry (LC-MS) and matrix assisted laser desorption ionization – time of flight (MALDI-TOF). The individual pure peptides with superior anticancer activity was subjected to characterization and amino acid sequencing. The anticancer and anti-ACE-I soy peptides obtained would have a wider application in supplements, food products, and medicinal categories.

The specific objectives of this research proposal are

1. Evaluation of protein and amino acid profile of selected Arkansas grown soybean lines.
2. Extraction of protein isolate (SPI) and production of gastro-intestinal resistant protein fractions with specific molecular sizes: 10-50Kda, 5-10Kda, and <5Kda.
3. Determination of ACE-I inhibitory properties of the GI resistant protein fractions.
4. Investigation of anticancer activities of protein fractions on human blood, breast, colon, lung, liver and prostate cancer cell lines.
5. Separation, purification and characterization of single pure peptides for enhanced bioactivities.
6. Mass spectrometry analysis for amino acid sequencing of single pure peptide.

CHAPTER 2

Literature Review

Soybean production

Soybeans are one of the major oilseed crops in the United States accounting for about 90 percent of U.S. oilseed production. Large-scale production of soybeans started in the last century in the United States as the area planted expanded rapidly. Soybeans are the second-most-planted field crop in the country with 83.7 million acres planted in 2014 which is the highest so far (Good, 2015). It is estimated that 30 to 40% of U.S. grown soybeans are exported (Ross, 2015). Increased planting flexibility, steadily rising yield improvements and low production costs favored growth of soybean acreage in the U.S. Greater than 80 percent of soybean acreage is concentrated in the upper Midwest states in the U.S., although significant amounts are still planted in the historically important areas of the Delta and Southeast. Stocks of soybeans are expected to be at an eight year high this current season owing to improved management practices, and enhanced product utility for commercialization.

The state of Arkansas ranks 10th in soybean production annually, where they are cultivated in more than 41 of the state's 75 counties (Arkansas Soybean Promotion Board, 2014, Ross, 2015). Soybean production, since 1980s, has been one of the largest agricultural enterprises in Arkansas with annual acreage of approximately 3.2 million (Ross, 2015). The crop generates an annual revenue of \$1.7 billion with an average of 48 bushels per acre (Ross, 2015). Soybean meal is a by-product obtained after oil extraction from the seed and is a good source of protein (Stein *et al.*, 2008). Annually production in the U.S. is 40 Million Metric tons and approximately 90% of it is used as a high protein component in livestock feed (Soya tech, 2015). However, in recent years its high value protein content has gained popularity in human diets as it

provides the basis for soy milk and vegetable protein. It is also used as a protein rich ingredient in lieu of its functionality in food products.

Soybean seed components

Soybean is high in protein content, and 70% of the crop value is attributed to this component. The protein content ranges between 37-42% in dry weight depending on the genetic make-up and the crop location, while certain pure lines have up to 50% protein (Krishnan *et al.*, 2007). Soybean seed is composed of 8% hull, 90% cotyledons, and the 2% hypocotyls as shown in figure 1. The cotyledons contain about 40% protein, 20% oil, 35% carbohydrate, and 5% minerals in the form of ash.

In the seed the protein is held in discrete sites for storage, and this is estimated to be at least 60–70% of the total seed protein. Proteins in legumes belong to the globulin family called leguminins (11S) and vicilins (7S), or glycinin and beta-conglycinin, which account for 65%–80% of total soy proteins (Yaklich, 2001). Several products are made from soy and its protein, especially in the Asian cuisine and it is regarded as a healthy food ingredient. Apart from proteins, soy contains valuable constituents like isoflavones, oligosaccharides, and germ plant sterols, which have been studied for their health benefits.

Benefits of soy consumption

Soy product consumption has increased due to their large beneficial properties such as being free of cholesterol, gluten, and lactose, making them suitable for allergen-intolerant consumers and vegetarians. Soy has certain health benefits in conditions like cancers, cardiovascular diseases, hypertension, and hormonal imbalances (Nair *et al.*, 2002). The seeds are the economic part of the soy plant. Key health benefits of soybeans are due to the high

quality protein, essential fatty acids, vitamins, minerals, isoflavones, saponins, and dietary fiber in the seeds (Perkins 1998, Ukwuru 2003, Leblanc et al 2004). Soy contains certain phytochemicals called Isoflavones, which have potential medicinal benefits like decreasing the symptoms of menopause, reducing the risk of certain cancers, improving bone strength, lowering of cholesterol, and preventing kidney disorders (Leblanc *et al.*, 2004). Soy seeds are ground to make soy flour, which is used to prepare various food products. The use of soy in human nutrition has increased significantly. There are numerous products on the market which are based on soy or contain soy ingredients. Protein products for food uses include defatted flakes, grits and flour, protein concentrate, protein isolate, and textured protein products. Soybeans can also be used to produce whole bean products.

Soybeans are known for their high protein content and are popular among vegetarians and the poor. They have the advantage of being a cheaper source of high quality protein and nutritionally equivalent to animal protein. By the turn of the millennium, the popularity of soy-foods has increased among health-conscious individuals. Soy products were consumed at least once a week in 31% of U.S. population (United Soybean Board, 2014) with retail soy-based food industry worth \$4.5 billion according to Soyfoods Association of North America (2014). Interest in soy and soybean constituents is due to the potential health benefits in a variety of areas including cancer, osteoporosis, and coronary heart disease (CHD) (Badger, T. M., et al., 2005, and Wang, W. and de Mejia, E. G., 2005). Soybeans are a source of dietary peptides known for the anti-hypertensive, anti-cholesterol, and antioxidant activities. The soy peptides also have a major role in preventing cancers like colon, breast, prostate, and lung (Su *et al.*, 2007, Hernandez-Ledesma, 2009).

Significance of soybean cultivars

Breeding newer varieties of soy is essential to produce cultivars which are resistant to pests, tolerant to diseases, produce higher yields in terms of protein and oil, and sustain in harsher environment. Genetic engineering techniques are extensively employed in the soybean breeding programs in the United States ever since the introduction of the crop into the country. There has been extensive research in the academic and industrial fields in order to produce better quality soybean especially with high protein content, since soy protein has been a source of amino acids which are required for human growth and development. Hutcheson line of soy, commercially cultivated in the United States is resistant to certain viral diseases of soybean viz., Soybean Mosaic Virus (Wang *et al.*, 2005). The protein content is around 38 to 40 %. R95-1705 is a breeding line with parentage that includes Hutcheson and a high protein line (~ 45 %) from USDA. It is being grown on a trial basis for the tofu and soy sauce market (Medders, 2004). Soybean germplasm line 'S97-1688' (Reg. no GP-300, PI 633736) has value as a parent because of its competitive yield potential, higher protein content of 45- 46 %, and broad resistance to populations of soybean cyst nematode, *Heterodera glycines* Ichinohe (Anand *et al.*, 2004). USDA and Agriculture research station at Raleigh, North Carolina developed N98-4445A, a new soybean germplasm developed through traditional breeding methods by scientists. The oil extracted from N98-4445A contains high levels of monounsaturated oleic acid, which is stable at room temperatures and does not require the hydrogenation for hardening, a property useful to prepare salad dressings, margarines, breakfast bars and baked food products (NC state soybean producers association, 2004, available at: http://www.ncsoy.org/html/may_2004.htm). Generally, the seeds are dried after harvesting from the field, and stored at cooler temperatures before further processing. The oil extracted from the soybean is used for cooking food products in the

form of vegetable oil, while the meal that remains is utilized to isolate the protein from the carbohydrates, and other constituents. This would make the soybean meal an excellent source of protein and carbohydrates, especially as an ingredient in cattle, equine, or poultry feed formulations.

High oleic acid soybeans

Soybean oil consists of palmitic, stearic, oleic, linoleic, and linolenic fatty acids. Out of these, linolenic acid (LA) is categorized under the polyunsaturated fatty acid (PUFA). It is a major PUFA present in soybean oil, which is crucial for LDL-cholesterol lowering effect (McKevith, 2005). Oleic acid is the monounsaturated fatty acid found abundantly in olive oil, and is less prone to rancidity. It has a longer shelf life than linolenic acid. Around 25% of oleic acid is present in conventional soybean oil, while linolenic acid is 7%. Although PUFAs have a beneficial effect on the tissue nutrition, they have detrimental effect when they get rancid or spoiled. Partial hydrogenation is used to increase stability and shelf life making soybean oil more edible; however, this process will ultimately produce trans-fats, which act as pro-oxidants and become carcinogenic when consumed by the human beings. Oleic acid does not show these effects and hence, studies of producing higher amount of oleic acid have been undertaken.

Genetic modification of soybeans to produce high oleic acid in the seeds is done by incorporating the gene into a bacterial cell and its transformation into the plant cells by recombination and DNA cloning techniques (Australia New Zealand food authority, 2001). Desaturase enzyme is required for the synthesis of linolenic acid in soy. Insertion of a second copy of the desaturase gene causes gene silencing, where both copies of the fatty acid desaturase gene are switched-off, and prevent the synthesis of linolenic acid. Due to lowered linolenic acid

production, the oleic acid accumulates in the developing soybean seed. Soybean bred to have low levels of linolenic acid would be replaced by higher quantities of oleic acid. Hence, soybean oil that is extracted from these seeds is less likely to deteriorate or go rancid, and therefore does not need to be hydrogenated. Researchers in the academic institutions, public and private soybean breeders in the United States released soybean varieties with as low as 1% linolenic acid (Australia New Zealand food authority, 2001). Soybean breeders are working on mid-to high-oleic acid soybean varieties with genetic modifications to develop cultivars that consistently produce an oil with greater than 50% oleic acid, called mid-oleic, and hope to combine the elevated oleic acid trait into their varieties with 1% linolenic acid that are grown commercially in the Midwest.

Proximate composition of Soybean flour and meal

Soybeans contain about 40% protein and are used in foods, livestock feed, and oil production, and are a source of other healthcare products, such as sterols and vitamin E. Since, soy protein contains almost all the amino acids that are essential to human nutrition; it is a great substitute for animal protein. Removal of the lipid content is the first step in preparing for extraction of proteins from the flour. Defatted soy flour contains 17% soluble and 20% insoluble carbohydrates and is an abundant and relatively inexpensive source of proteins (Perkins, 1998). The oil is removed from the soy seeds by extrusion method, or by solvent extraction. The soybean meal is collected to extract the protein. Solvent extraction is the most common procedure in the U.S. for separation of lipid from soybean flour. It is 100% extraction-efficient, and is suitable for handling large volumes of soybeans (Bargale *et al.*, 1999).

The composition of a typical solvent-extracted soybean-meal is given in Table 2.1. Soybeans from the northern and western soybean growing states contained 1.5-2% less protein and 0.2-0.5% more oil than soybeans from southern states due to the environmental impact on the yield (Grieshop *et al.*, 2003).

Tables 2.1. Composition and nutrient content of 100 g soy flour (USDA, 1986).

Type	Moisture (g)	Protein (Nx5.71)	Fat (g)	Carbohydrate (g)	Crude Fiber (g)	Calcium (mg)	Iron (mg)
Defatted	7.3	47.0	1.2	38.4	4.3	241	9.24
Full fat	5.2	34.5	20.6	35.2	4.7	206	6.37
Type	Zinc (mg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin B6 (mg)	Folacin (mcg)	Sodium (mg)
Defatted	2.46	0.70	0.25	2.61	0.57	305.4	20
Full fat	3.92	0.58	1.16	4.32	0.46	345.0	13

Mechanical oil expression is another method with an extraction efficiency of around 70% and supplies to a specific niche market (Bargale *et al.*, 1999). Improvement in the economics of mechanical oil extraction rate can make it viable on a large scale in the soybean industry in the United States. The defatting techniques of soy protein isolates can modify their functionality. N-hexane has been used extensively in soy processing to remove fat from the soy flour. Other alternative techniques such as non-alcoholic aqueous extractions have also more recently studied (L'hocine *et al.*, 2006). Improvement in processing and functional characteristics has resulted in an ever-increasing demand for soy protein ingredients from soybean meal.

Soy meal is a byproduct of soy oil industry and is the raw material for extracting soy protein. Research in alternate fuels and the advent of bio-diesel, a product that needs soy oil for preparation, leads to increased production of soybean meal. Since, being a byproduct, it is a less expensive source for producing proteins rich in essential amino acids. Soybeans from the northern and western soybean growing states contained 1.5-2% less protein and 0.2-0.5% more oil than soybeans from southern states (Grieshop *et al.*, 2003). The study also showed that crude

protein concentrations of soybean meals samples collected from the southern U.S. maturity zones were higher than those collected from the northern maturity zones. The results from a study on the variations in protein and oil content in soy also prompted environmental growth conditions to influence the protein concentrations (Hurburgh *et al.*, 1990), where, U.S. grown soybeans showed consistent state and regional differences.

Soy protein concentrates are available in different forms; granules, flour and spray dried. Because they are very digestible, they are well-suited for children, pregnant and lactating women and the elderly. Most fiber of the original soybean is retained in the soy protein concentrate and is widely used as functional or nutritional ingredient in a wide variety of food products. Soy protein concentrate (SPC) is about 70% soy protein and is basically soybean without the water soluble carbohydrates, which are removed from de-hulled and defatted soybeans. SPC is used in meat and poultry products to increase water and fat retention. Due to the presence of high protein content, it is used in infant foods to improve nutritional values with more protein, and less fat. They are also used in pet foods, milk-replacers for calves, pigs, and even used for some non-food applications.

Soy protein is also present as an isolate where it is separated from the low molecular weight polysaccharides present in the flour. Soy protein isolate (SPI) is prepared by chemical or enzymatic hydrolysis of the soy protein (Morita and Kiriyaama, 1993, Shih and Daigle 2000, Paraman *et al.*, 2006). The alkaline hydrolysis involves solubilizing the protein in water by increasing the pH to a range of 8 to 10. The insoluble starch is separated by centrifugation and the protein is precipitated by bringing the suspension to iso-electric pH of 4.5. Alpha-amylase is used to digest the starch and separate the protein from the soy flour in suspension in the enzymatic hydrolysis method.

Soy protein has been studied for the significant health benefiting activity in human beings in controlling diseases like hypertension, and heart disease. More than performing different metabolic and regulatory functions, proteins from food sources have been identified to be antagonists for many disease states like cancer, hypertension, oxidative stress, inflammation, and diabetes (Meyers *et al.*, 2003, Kim *et al.*, 2004, Nam *et al.*, 2005). The protein is broken down into peptides by enzymatic methods. In general, naturally occurring peptides are classified based on the function they perform. Some are classified as signal transduction components, others gene regulators, while some others serve as components mediating metabolism, translation, folding and degradation (Monowar et al 2004).

Amino acid analysis of proteins

The composition of amino acids in proteins can be determined by complete hydrolysis of the protein into its amino acid residues. The most common method is by acid hydrolysis followed by performic acid oxidation for cysteic acid and methionine sulphone. Alkaline hydrolysis can be used to determine the amount of tryptophan, an essential amino acid, in the protein. However, significant destruction of amino acids occurs during acid hydrolysis of protein from in food material which is more evident with the essential amino acids: methionine, cystine, lysine, threonine, and tryptophan. Specific hydrolytic techniques would need to be carried out for different foodstuffs as their proteins differ widely in amino acid compositions. Amino acids are released and destroyed at different rates that depend upon the amino acid composition and characteristics of the proteins in the sample.

Chromatographic techniques including High Pressure Liquid Chromatography (HPLC) and Gas-liquid chromatography are used for detection of amino acids and their quantification.

Pre-column derivatization with Dansyl chloride (5-dimethylamino-1-naphthalene sulphonyl chloride) is required which produces fluorescent dansyl derivatives that can be separated by a reversed phase column chromatography. Ion exchange chromatographic separation of peptides has been demonstrated to be more effective when followed with HPLC that uses various non-polar stationary phases, for separation of non-polar amino acid derivatives (Molnar and Horvath, 1977).

Preparation and bioactivity of peptides from various food sources

Soy protein contains all the essential amino acids, and hence is a good source of bioactive peptides when exposed to hydrolysis by the gastro-intestinal enzymes. A thorough understanding of the soy protein and its derivatives is possible when we digest them with the endoproteases, to produce the short chain peptide fragments, and evaluate them for the functionality. It is also important to discover new peptides with health benefits in soy-hydrolysates. The identification of novel bioactive compounds will contribute towards the development of functional foods that can be used to enhance health and quality of life.

Proteins are made of amino acids, while peptides are chains of amino acids cleaved-out from the main protein molecule. They may have amino acids ranging from 3 or 4 to over 50 amino acids. Enzymatic hydrolysis, digestion in the gut, and food fermentation are the three methods in which protein fragments or short amino acid chains are produced. Peptides might occur naturally in foods, can be the products of human digestion, or can be obtained in the lab through enzymatic hydrolysis. Controlled hydrolysis of the soy protein has the advantage of producing peptides with certain number of amino acids that will enhance their functionality.

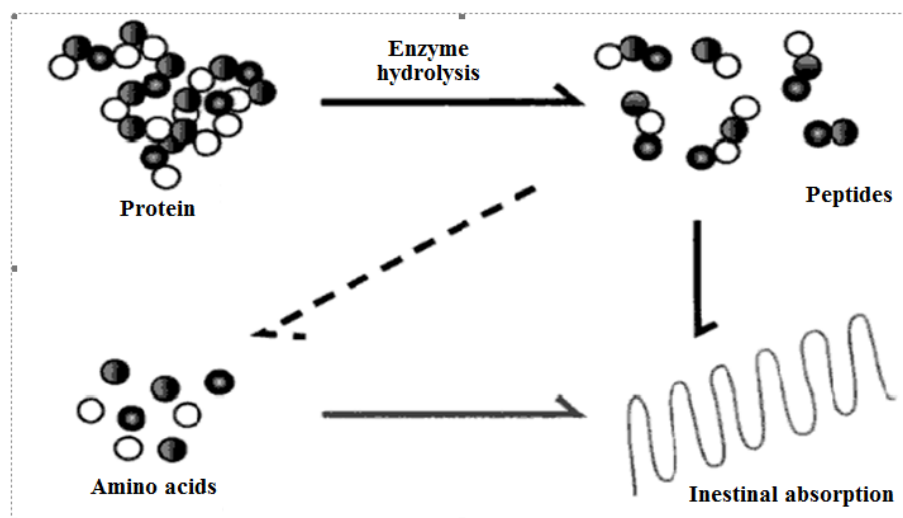


Figure 2.1. Breakdown of proteins into peptides and amino acids by enzyme hydrolysis (Sugano, 2006).

Proteins like whey, casein, soy, and those obtained from certain fruits and vegetables are known to lower disease progression due to their bioactivity. Many of these are subjected to proteolysis to yield peptides in various size ranges. These are called dietary peptides which also exhibit functional properties like the proteins (Figure 2.1). Foods like cereals, milk, vegetables and fruits also contain many bioactive proteins/peptides present in their tissues and have different functions (Korhonen *et al.*, 2003, Antonio, 2003). Food-derived bioactive peptides from dietary proteins commonly contain 2–9 amino acids, which may be extended to 20 or more amino acid units (Kitts D. D, and Weiler K., 2003). The food industry has shifted towards using biologically active proteins and peptides, while food researchers are considering different bioactive substances of food origin as health enhancing ingredients for use in foods commonly referred to as functional foods or nutraceuticals (Mine Y, and Shahidi F., 2006, and Shimizu and Dong Ok Son, 2007). Food producers can make health claims about their products if they can produce sufficient scientific evidence.

The research on bioactive proteins and peptides has some common objectives like, providing evidence, and giving human-study basis for the reduction of disease risk claims on the labels, scientific design for proving the persistent effect of the food or pharmaceutical preparation, and evaluating the risks of consumption, including allergic potential. Regarding the discovery of novel peptide drugs, the chemical properties of food-derived peptides and proteins can be readily modified in systematic ways for drug design and development. Small changes in the structure can lead to dramatic changes in bioactive potency and function, and these peptides are generally less toxic than other organic 'natural molecules'. Future research should focus on establishing novel production technologies and understanding the interaction of different bioactive proteins and peptides with multiple components during production and within a complex food of an overall diet. There are many unknown factors regarding to physicochemical and biological interactions of bioactive ingredients, which may result in inactivation or in synergisms and antagonisms. Understanding the mechanisms of possible interactions is important to develop an adequate packaging system, like micro-encapsulation, to enable the improved delivery and protection of bioactive ingredients. Research continues to discover novel bioactive proteins /peptides, functions and health benefits - all of which reveal the striking potential for natural selection through evolution to produce food molecules that act beyond simply providing nutrients. Discovering these benefits and potential applications remains a great challenge for both nutrition research and pharmaceutical design.

The processing of protein into peptides in the GI tract greatly increases their healthful effects by exposing active groups within the amino acid chain. Soy peptides in recent studies have shown in to be most promising anticancer agents. Found in major readily-available foods in the market, these peptides are an accessible component to healthy living. In 1999, the Food and

Drug Administration approved a health claim for the cholesterol-lowering properties of soy protein (Pena-Ramos E. A., and Xiong Y. L., 2002) and soybeans are known to contain several bioactive components (Kim S. E. *et al.*, 2000). Proteins in soybean have hypo-cholesterolemic, anti-atherogenic, and body fat reducing properties, while the soy peptides also reduce body fat, and are readily absorbed into the blood (Michihiro Sugano, 2006). As more results are reported on the biological activities of such proteins, little is known about the mechanism by which proteins exert these functions, or the basis for each protein to function so uniquely. To understand better a study is required on peptides that these proteins breakdown into when digested with proteases. Such a study would help in determining what portions of the protein constitute the functional aspect. Soy peptides are known for their bioactivity in the organ tissues and protect the body from diseases like cancers, tumors, mutations, and high blood pressure. Recent advances in science have brought various techniques of analyzing the beneficial effects of the soy peptides, and to determine their amino acid sequence. These peptides would be synthetically produced and incorporated into foods to deliver the health benefits.

The bioactive peptides produced by soybeans possess diverse and unique health benefits. These can be effective in the prevention of age-related chronic disorders, such as cardiovascular disease, cancer, obesity, and decreased immune function. Bioactive peptides are released from dietary proteins by either gastrointestinal digestion or by the processing of foods. It is known that during gastrointestinal digestion or food processing, these peptides are released from the parent protein and act as regulatory compounds with hormone-like activities (Korhonen H, and Pihlanto, 2003). Microbial fermentation is an efficient way to produce bioactive peptides which can be released by the action of enzymes derived from microorganisms (Korhonen H, and Pihlanto A., 2003). Interest in bioactivity of fermented soy protein rich products including milk,

natto, tempeh, soy sauce, soy paste has grown in recent years (Wagar *et al.*, 2009, Rho *et al.*, 2009, Kwon *et al.*, 2010). Tryptic hydrolysates of soy protein produce immuno-modulatory peptides. Kitts and Weiler (2003) showed that these peptides stimulate super-oxide anions, which trigger nonspecific immune defense systems. Evidence suggests that these peptides are absorbed by the gastrointestinal system, and exert their action on specific target organs in humans. Research and clinical trials have demonstrated the biological activities of peptides, but the specific mechanism of action requires further investigation.

Research in bioactive compounds from foods that provide nutrition and health benefits since the late eighties have opened new frontiers in the field of food and nutrition (Mine Y, and Shahidi F., 2006). These substances, which are both nutrient and non-nutrient, produce regulatory effect in the organ tissues of the human body, particularly to control a disease or to elicit a specific physiological function. Many bioactive peptides are inactive, and require enzymatic proteolysis for release from their protein precursors. Once activated, these bio-peptides exert activities such as ACE-Inhibitory, immuno-modulatory, antimicrobial, mineral binding, and anti-mutagenicity.

Shimizu and Dong Ok Son (2007) studied the effect of food-derived peptides and their intestinal functions while getting hydrolyzed by digestive enzymes in the intestinal tract. The absorption of the peptides intact is important for their action in their target organs, while the intestine-modulatory peptides can express their functions in modulating the intestinal epithelial cell functions. These peptides are studied to cause prevention of certain intestinal diseases. Certain peptides showed cytotoxicity which leads towards a cancer protective effect that could exist for such bioactive peptides (Hartmann R. *et al.*, 2007).

Antihypertensive activity and sources

Heart disease is number one cause of diseases-related deaths in the United States and it affects both children and adult population (Mozaffarian *et al.*, 2014). According to the American Heart Association and the Center for Disease Control and Prevention 610,000 people die of heart-related diseases in the U.S (CDC, 2015). Hypertension is a major risk factor for coronary heart disease and the most important factor for stroke related deaths (National Heart, Lung and Blood Institute, 2014). Individuals less than 50 years old are prone to increased cardiovascular risk due to elevated blood pressure. Increase in blood pressure is known to be significant due to aging, but diet and other environmental factors cause hypersensitivity due to alterations in biochemical pathways.

Proteins from foods have been shown to possess anti-disease properties (Mine and Shahidi, 2006). Most bioactive peptides are antihypertensive and show their activity by inhibiting angiotensin-I converting enzyme (ACE-I). ACE-I belongs to a nonspecific dipeptidyl-carboxypeptidase group of enzymes which is associated with the regulation of blood pressure. It modulates the rennin-angiotensin system by converting the decapeptide angiotensin I into angiotensin II, a potent vasoconstricting octapeptide. This leads to an increase in blood pressure, and hence, inhibition of the ACE-I will result in an antihypertensive effect (Shin Z. I. et al., 2001). Research found ACE-I inhibitory bioactive peptides in soy-protein enzyme hydrolyzates. In vivo experiments on 'spontaneously-hypertensive' rats had lowered blood pressure when the peptide fractions were administered orally. Alcalase digests of soy protein also produced antihypertensive peptides in the same experiment (Wu J., and Ding X., 2001). There was a significant ($P<0.05$) decrease in systolic blood pressure in the rats when oral doses of these peptides were given in a dose-dependent manner. However, higher doses (1000 mg/kg of body

weight/day) did not produce any effect on the blood pressure of normo-tensive rats. Fermented soybean products are a good source of ACE-I inhibitory bioactive peptides. Research found that traditional Asian fermented soy foods, such as soybean paste (His-His-Leu), soy sauce, natto, tempeh, and chunggugjang, a traditional fermented Korean soybean product have ACE-I inhibitory peptides (Okamoto A. et al 1995, Gibbs B. F. et al 2004, Potter S. M., 1995, Korhonen H, and Pihlanto, 2003).

Healthier and natural ACE-I inhibitory peptides can be prepared from daily dietary food proteins. These novel functional food additives will replace the expensive and health diminutive ACE-I inhibitor drugs. Anti-ACE-I activity of has been investigated in casein (Maruyama et al 1985), fish muscle (Kohama et al 1998), soy sauce (Okamoto et al 1995), and rapeseed (Marczak et al 2003), from the respective peptides either in vitro or in vivo. Potter, in 1995, reported that hyperlipidemic or hypercholesterolemia lesions that cause arteriosclerosis and hypertension are preventable by consumption of soybean protein. Fermented products like soybean paste (Ahm et al 2000) and soybean hydrolysate (Yu et al 1996) were shown to have exerted an inhibitory activity on ACE-I in vascular tissue in vivo. Four ACE-I inhibitory peptides isolated from the peptic digests of proteolysis of glycinin were engineered using varied proteases to harness peptides that exhibit ACE-I inhibitory activity. Accordingly, the Protease P hydrolysate contained a suite of very potent ACE-I inhibitory peptides that were subsequently fractionated and purified. A significant fraction of the ACE-I inhibitory activity could be ascribed to a pentapeptide, Val-Leu-Ile-Val-Pro (VLIVP). Considering the diversity and complexity of the different protein types of soybean, soy protein provides an abundant source of biologically active peptides. Many potential antihypertensive peptide sequences are embedded in the available protein sequence data for glycinin, the major storage protein and 11S globulin of soybean. This

potential is yet to be realized. There exists therefore a great potential for releasing these bioactive peptides from their latency using proteases of varied specificity. In the present investigation, proteolysis of glycinin was engineered using varied proteases to harness peptides that exhibit ACE-I inhibitory activity.

There have been reports on antihypertensive food peptides with bioactivity other than ACE-I inhibition. These peptides were found to possess antioxidant, vasodilator, and opioid activities (Yoshikawa et al 1994, Fujita et al 1996, Sipola et al 2002, and Kuono et al 2005). Peptides derived from egg-white, and their synthetic analogues exerted non-ACE-I-mediated vasodilator effects in controlling hypertension (Fujita *et al.*, 2000). In other studies, pepsin hydrolyzed egg white inhibited ACE-I *in Vitro* and exhibited antihypertensive effects in spontaneously hypertensive rats *in vivo* (Miguel et al 2006). Peptides from egg whites significantly reduced the vascular resistance and produced blood-pressure-lowering effects in hypertensive rats, independent of ACE-I inhibition (Fujita et al 2000). Although more studies are necessary to clarify the mechanisms responsible for the antihypertensive and vasodilator properties of these sequences, our findings provide new evidence for the potential of egg-white peptides as functional food ingredients with therapeutic benefits in the prevention and treatment of hypertension and other associated disorders. The ACE-I inhibition has a major role in anti-hypertensive activity resulting in reduction of heart disease; the peptides exerting such bioactivity are favorable for production either by enzyme hydrolysis of protein isolates or artificial synthesis. The bioactive peptides have varied activity depending on the organ tissue in to which they are exposed. Hence, these peptides can be studied for additional bioactivity and health promotion, since research supports such a possibility. Various sources of bioactive peptides and their activities are listed in Table 2.2.

Table 2.2. List of proteins and their products with anti-ACE-I activities.

Anti-ACE-I agent	Source	Reference
Peptide fractions	Fish (Tuna)	Lee <i>et al.</i> , 2010
Peptides	Spinach	Yang <i>et al.</i> , 2003
Peptide	Tuna muscle	Qian <i>et al.</i> , 2007
Peptide fractions	Yogurt	Donkor <i>et al.</i> , 2007
Peptides	Hydrolyzed corn gluten	Yang <i>et al.</i> , 2007
Protein Hydrolysates	Pipe fish (<i>Syngnathus schlegeli</i>) muscle protein	Wijesekara <i>et al.</i> , 2011
Proteins	Casein	Maruyama <i>et al.</i> , 1987

Various cancers and anti-carcinogenic compounds.

Colon cancer

Colon cancer or colorectal cancer is the third most common cancer and the third leading cause of cancer related mortality in the United States (American cancer society, 2015). Eleven percent of total cancer deaths are due to colorectal cancer, especially in men who are 50 years and beyond. It has relatively low incidence in women. Most colon cancers develop as a tissue growth into the center of the colon or the rectum called Polyps. Certain types of polyps normally tend to become cancerous and are called adenomas. Ninety five percent of colorectal cancers are adenocarcinomas. Evidence shows that dietary components are the major factors in the cause of the colorectal cancer. Animal fat, red meats, chocolate, alcohol and refined cereals are the foods that have been identified to promote high incidence of colorectal cancers in the Western societies (Sandler RS, 1996, Potter JD, 1996, Levi *et al.*, 1999). Number of clinical and epidemiological studies provide clues that these food compounds may act as suitable markers or dietary determinants that when modified and prepared in a bio-functional form can serve as bioactive compounds in reducing the incidence of colorectal cancers (Cancer statistics, 2004)., whereas fruits, vegetables, fiber, whole grains and their compounds like folate and calcium have been

identified as protective agents against not only colorectal but also other cancers (Levi *et al.*, 1999). Apoptosis is found to be the best natural defense against cancer. The molecule, P53 induces apoptosis in some cell types when over-expressed, and is in fact considered invaluable to induce apoptosis in states of DNA damage (Sherr, 1996). The P53-induced apoptosis activity is common since P53 can either activate death genes like the Bax, or suppress survival genes like the Bcl-2 (Miyashita *et al.*, 1994). Both these are considered molecular markers to detect apoptosis.

Breast cancer

Generally, most breast cancers originate in the ducts (ductal carcinoma) while others are found in lobules (lobular carcinoma) or in other parts of the breast tissue. The common form of breast cancer is the invasive ductal carcinoma that develops and penetrates the duct wall before invading the fatty tissue of the breast before spreading to other parts of the body. According to American Cancer Society invasive type of breast cancer accounts for about 80% (DeSantis, 2013). It is the second leading cause of cancer-related death in women and over 3 million women have been treated in the U. S. so far for breast cancer (Siegel, 2014).

Lung Cancer

Lung cancer develops along the lining of the bronchi, and with time, causes precancerous changes within the lung. It is the most leading cause of cancer death in men and women in the United States due to the habit of cigarette smoking among the population (American Cancer Society, 2015). Formation of chemicals for new blood vessels allows for tumor formation and denotes true cancer development. At an advanced stage the cancer cells break away and spread to other tissues, which is called metastasis and 85% of all lung cancers are non-small cell type (American Cancer Society, 2015). According to the American Cancer Society (2015) other

classifications of lung cancers include squamous cell carcinoma, adenocarcinoma, and large-cell undifferentiated carcinoma. For the year 2015, it is estimated that 221,200 new cases of lung cancer (both small cell and non-small cell) will be diagnosed and 158,000 deaths are predicted.

Liver cancer

Liver cancer is the leading cause of cancer related deaths in the world but it is less common in the United States among major cancers discussed here (American Cancer Society, 2015) with 35,660 new cases that are expected to be diagnosed and 24,550 deaths are predicted in 2015. It is more common in men than in women and is caused by mutations in the liver cells resulting in several types of malignant (cancerous) and benign (non-cancerous) tumors. However, benign tumors, most common type is hemangiomas, do not grow into nearby tissues or spread to distant parts of the body. Hepatocellular carcinoma is the most common type which forms cancer nodules in the liver and is the most common type seen in the U. S.

Prostate Cancer

Prostate cancers start developing in the prostate gland with very small morphological changes occurring to the gland cells. This type of cancer is also called adenocarcinoma. The changes that occur are called prostatic intraepithelial neoplasia (PIN) that can be either low-grade or high-grade. A large number of cancerous cell proliferations are expected in the case of high-grade differentiation (American cancer society, 2006). Prostate cancer is the most common non-skin cancer in the United States. It is the second most cause of cancer death in men, first being lung cancer. The American Cancer Society has predicted approximately 220,800 new cases and 27,540 deaths due to prostate cancer in 2015 in the United States (American cancer society, 2015). There is not much involvement of food proteins or peptides with prostate cancer research as yet, as they do with other cancers.

Blood cancer

Unregulated proliferation of abnormal blood cells are characterized as a group of diseases called blood cancers. The disease affects the production and function of blood cells in the bone marrow, which is the site for stem cells that mature and develop into three types of blood cells: red blood cells, white blood cells, or platelets. In most blood cancers the abnormal or cancerous cells prevent blood from performing many functions, including fighting infections or preventing serious bleeding. According to American Society of Hematology there are three major kinds of blood cancers:

1. Leukemia is caused by the rapid production of abnormal white blood cells which impairs the bone marrow to produce red blood cells and platelets and reduces the ability to fight infections.
2. Lymphoma affects the lymphatic system, which removes excess fluids from the body and produces immune cells. Abnormal lymphocytes become lymphoma cells, which multiply and collect in the lymph nodes and other tissues thereby disrupting the immune system.
3. Myeloma affects the plasma cells which are white blood cells that produce disease- and infection-fighting antibodies.

The American cancer society estimates 54,270 new cases of leukemia in 2015 and 24,450 deaths are expected due to the disease in the United States. The leukemia's are classified into four main groups according to cell type and rate of growth: acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myeloid (AML), and chronic myeloid (CML). Chemotherapy is the only treatment for blood cancers and the expected survival ranges between 25% (AML) to 84% (CLL) depending on the cancer state and disease progression.

Control of cancer using food components

Researchers have hypothesized several mechanisms of action using specialty compounds in mitigating breast cancer. Aiding chemoprevention using vitamins and other compounds to reduce the risk of developing cancer, block or suppress malignant transformations or disease progressions has gained considerable attention in the recent years. Some of the possible biochemical mechanisms include apoptosis, arresting cell cycle progression, and also interfering with metabolism. Several food products are known to contain biomolecules that aid in alleviating the disease progression by preventing cancerous cell proliferation. Agricultural products including fruits and vegetables are considered good sources of anti-cancerous/anti-proliferative agents (Hocman G, 1989). For example, breast cancer cell lines when tested with the juice extracts of Brassica family exhibited anti-proliferative effects signifying the presence of constituents within the vegetables can prevent cancer progression (Brandi et al, 2005). Proper modification and application of these products can replace costly drugs or other invasive treatments of cancer.

There are several compounds documented and thought of as being able to delay or reduce cancer progression. Tricin, for example, present in rice bran is a flavone that is shown to cease colon cancer cell proliferation. Furthermore, it inhibited cyclooxygenase activity, reduced prostaglandin production and thus interferes in intestinal carcinogenesis in mice (Cai *et al.*, 2005). Certain chemo-preventive phenols obtained from brown rice were shown to inhibit growth of both colon and breast cancer cells. Most of the functions that rice bran elicits are attributed to its non-protein components. However, recent studies have shown that a peptide derived from rice bran to possess activity against cancer (Kannan *et al.*, 2010, Li *et al.*, 2014a and 2014b), Alzheimer's and obesity (Kannan *et al.*, 2012). The newly discovered soybean

peptide lunasin is shown to be an anticancer agent even though evidence of an effect of soy peptides on oncogenesis still requires groundwork (Kobayashi H. et al., 2004). Suppression of ovarian cancer cell invasion by blocking urokinase upregulation was attributed to Kunitz trypsin inhibitor, a soy peptide with anticancer properties in their study by Xiao R. et al (2005). Papain catalysis of Tuna dark muscle by-products resulted in a 12 amino acid peptide that showed anticancer effect (Hsu *et al.*, 2011). Kim S. E et al (2000) studied the inhibition of tumorigenesis by a soy protein isolate diet due to enhanced production of somatostatin which caused anti-proliferation effect. Several low and high molecular weight peptides derived from giant squid (*Dosidicus gigas*) using proteolysis have been demonstrated to show cytotoxicity against MCF-7 human breast cancer cells (Aleman *et al.*, 2011). Peptides from other seafood sources including shrimp and snow crab proved to be sources of peptides with anticancer activities (Doyen *et al.*, 2011, Kannan *et al.*, 2011). Peptides obtained by thermolase hydrolysis of defatted soy protein, further purified with ethanol and fractionated by gel filtration chromatography, showed an *in vitro* cytotoxicity on mouse monocyte macrophage cell line. At 1 mg/mL, this fraction significantly affected cell cycle progression by arresting the cells in G2/M phases. Purification of these peptides with C18 HPLC resulted in 1157 Da nonapeptide (X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr) (González de Mejía E. et al., 2004).

A protein in blood with a tripeptide sequence (Arg-Gly-Asp) is commonly known to bind and exert biological functions like interference with cancer cell cycle progression (Yang et al, 2006). Peptides containing this particular sequence demonstrated positive anticancer activity by inhibiting tumor growth formation, induced apoptosis and cell cycle (G1 phase) arrest in breast cancer cell lines (Yang et al, 2006). A plant protein, calcaelin isolated from the mushroom *Calvatia caelata* was found to reduce breast cancer cell viability and progression (Ng et al,

2003). The differences in protein expressions during healthy and cancer state have been utilized by studies to identify and monitor these marker proteins for cancerous states to determine the disease progression (Roberts et al, 2004). A few of them include estrogen receptor-alpha, beta-caesin, bax, and cytokeratin 7.

Food sources have been studied for reducing lung cancer by deriving compounds that affect the cancer cell apoptosis and biochemical pathways that can lead to tumor stasis. Studies have found an association between consumption of large quantities of fish and a decreased rate of lung cancer (Takezaki et al, 2001). Certain chemical compounds, called cumestrans, present in green vegetables have been shown to suppress lung cancer disease states (Schabath *et al.*, 2005). Isoflavones present in a wide variety of food products including soybeans, yams and chickpeas are common anticancer agents in reducing the rate of lung cancer (Shimazu *et al.*, 2010). Certain proteins are also known to elicit anticancerous and apoptotic roles. Researchers have demonstrated apoptotic properties of milk on transformed cell lines where the protein responsible was identified as a multimeric form of alpha-lactalbumin (Hakansson et al, 1995). The lung cancer cells (A549) exhibited characteristic features of apoptosis while their viability was reduced by 98%. Animals fed with protein rich diets (casein) showed reduced pulmonary metastatic disease and tumor weight in other studies (Mahaffey et al, 1987).

Certain nutrients that are found in natural foods have been shown to reduce the incidence of developing prostate cancer, and also help slow down the disease progression. Again the nutrients present in fruits and vegetables serve important roles in mitigating prostate cancer. Of the many compounds present in fruits and vegetables, lycopene has been long considered to be an excellent fighter against prostate cancer. Lycopene is present in tomatoes, papaya, grapefruit, watermelons, and guava. The action of lycopene is attributed to its ability to scavenge free

radicals and serve as a powerful antioxidant (Hwang *et al.*, 2005). It has been shown that antioxidants slow cancer progression even at the stage when tumors are clinically detectable (Borek, 2004). Soy proteins have been studied to cause a possible reduction in the prostate cancer disease progression. With soy proteins it is only a possibility that they may inhibit prostate cancer since they affect tumor cell apoptosis and not inhibition of cancer cell proliferation. (Bylund *et al.*, 2000).

Chief compounds that are known to arrest prostate cancer cell proliferation are the flavones/isoflavones, soy proteins, substances like sulforaphane present in cruciferous vegetables. Bowman Birk inhibitor (BBI), a soy protein component, has been shown to suppress carcinogenesis in human prostate cancer cells and has also been the subject of promising clinical trials in cancer patients (Meyskens 2001, and Badger et al 2005). Large studies have shown that consuming at least two servings of tomato sauce per week confers a significantly lower risk of developing organ-confined prostate cancer, locally advanced disease, and metastatic disease (Bowen *et al.*, 2002, Giovannucci, 2002). Sulforaphane, a compound found in cruciferous vegetables such as broccoli and cauliflower, helps the body to repair the damage caused by cancer-causing substances, thereby helping to slow the cancer growth process (Myzak *et al.*, 2005). Isoflavones have been shown to influence the production, metabolism, and excretion of testosterone and estrogens, hormones that can play important roles in the development and spread of prostate cancer (Dillingham *et al.*, 2005). Increased consumption of soy products or isoflavones has shown some effect on slowing the rate of prostate specific antigen (PSA) rise in several studies, but not all patients responded in the same manner and the results have been inconclusive (Davis *et al.*, 2000).

Spinach flavonoids possessing antioxidant properties are known to cause chemoprevention of PCA using human PC3 cells. They are also known to have anti-proliferative, and anti-inflammatory properties. Flavonoids were found to arrest cell-cycle G1 phase, and cell-cycle prolongation that could possibly mediate the growth arrest of prostate cancer cells (Bakshi *et al.*, 2004). Peptides derived from spinach have also shown significant biological activities in human cell lines (Yang *et al.*, 2003). Another example of action of flavonoids originates from grape seed extract. Studies have shown that grape seed extract inhibits cancer cell proliferation and induces apoptosis of the same in nude mice (Agarwal *et al.*, 2002). Mechanism of action points to markers involved in the apoptotic signaling pathway. Markers like caspases were broken down upon treatment of cells with grape seed extract. The study concluded that the flavonoids from grape seed extract activated certain caspases and induces caspase mediated apoptosis (Agarwal *et al.*, 2002).

Role of proteins and peptides against cancer

Proteins and peptides have been shown to affect anti-proliferative effects on colon cancer cells, and these have been confirmed with animal studies. Predominantly flavones and isoflavones have been shown to possess anti-proliferative/anticancer effects on colon cancer cell lines. Cereals like rice or rice bran for example, has proteins, and other important components that inhibit cancerous cell proliferation (Kannan *et al.*, 2008, 2009, Li *et al.*, 2014). Amino acids may play an indirect role in impeding cancer induction. Many protein diets including soy protein contain non-essential amino acids that promote glucagon production. This in turn down regulates the activity of insulin and, produces IGF-1 (insulin like growth factor-1) antagonists. Additionally, the presence of low levels of essential amino acids in many diets (vegan diets) may decrease hepatic IGF-1 synthesis. This causes a negative effect in cancer formation. Hence these

diets when void of excess fat may prove protective against prostate, breast and also colon cancers that are linked to insulin resistance (McCarty *et al.*, 1999).

Although genetic factors are associated with the etiology of the disease and that they predispose individuals to colorectal cancer, much of this predisposition appears to be related to differences in dietary habits (Potter JD 1996). A number of *in vitro* studies suggest that proteins may be protective against colorectal and many other cancers. For example the whey protein has been proven to reduce the incidence of tumor formation in rats that were chemically induced to have colon cancer (Hakkak *et al.*, 2001). The same study compared the effect of casein protein as diet to that of whey protein in decreasing the tumor formation, and found that casein did not prove as effective in reducing the tumor formation. Also whey proteins have been shown to protect more effectively than red meat against chemically induced formation of aberrant crypt foci that are considered as markers for colon cancer, first detected in rodents in 1987 (Bird RP, 1987) and in Wistar rats (Belobrajdic *et al.*, 2003). Other proteins investigated for anti-carcinogenic properties of the colon include alpha-lactalbumin. Studies demonstrated cell growth during the initial 3 to 4 days at low concentrations of the protein alpha-lactalbumin (10-25 ug/mL). After the fourth day, cell proliferation had ceased with low viable cell counts suggesting probably a delayed apoptotic behavior (Sternhagen *et al.*, 2001).

Proteomics has greatly aided in the study of mechanisms of actions of several food components. Some of them include psychoactive compounds present in grape seed extract (Kim *et al.*, 2006), and anti-microbial peptides from cheese extracts (Losito *et al.*, 2006). Molecular targets on which these dietary components act have been elucidated using the colon cancer cells as a model with the help of proteomic techniques like the 2-dimensional gel electrophoresis, and mass spectrometry. The tryptic or proteolytic digests of proteins, biological actions of

compounds like quercetin on the growth of cancer cells was studied to the level of the proteome within the cell by Davis and Hord (2005). It was also found from the same study that quercetin altered the expression of 20 proteins at least 2-fold, and the proteins included several heat shock proteins and annexins. These proteins play significant roles in the physiology of apoptosis, metabolism, and gene regulation. (Wenzel *et al.*, 2004). Irrespective of the compound studied, a clear understanding of the mechanism of anti-cancerous or anti-proliferative effects can be visualized by studying the patterns of protein expression inside the cell that is subjected to study. That way proteomics proves useful.

As evident from the relatively fewer studies on proteins/peptides studied in relation to cancer therapeutics, compared to non-protein substances we emphasize the need to conduct more studies that help validate the bio-functional roles of proteins/peptides in cancer therapeutics. With advent of new processing strategies in rice to improve protein yields, there is a need to evaluate these rice bran proteins/peptides for their anticancer roles. The following table (2.3) lists the proteins/peptides that have been so far implicated in anticancer effects.

Table 2.3. List of proteins/peptides with anticancer activities.

Type of cancer	Anticancer agent	Source	Reference
Prostate	Proteins	Soy	Bylund <i>et al.</i> , 2000
Breast	Protein	Soy	Badger <i>et al.</i> , 2005
	Peptides with RGD sequence		Yang <i>et al.</i> , 2007
	Calcaelin protein	Mushroom (<i>Calvatia caelata</i>)	Ng <i>et al.</i> , 2003
	Peptide	Rice bran	Kannan <i>et al.</i> , 2010 and Li <i>et al.</i> , 2014
Lung	Alpha-lactalbumin	Milk	Hakansson <i>et al.</i> , 1995

Table 2.3. List of proteins/peptides with anticancer activities (cont.).

Type of cancer	Anticancer agent	Source	Reference
	Casein and other proteins	Animal	Mahaffey <i>et al.</i> , 1985
	Peptide fractions	Rice bran	Kannan <i>et al.</i> , 2008
Leukemia	Ribosomal inactivating proteins	Seeds of <i>Abrus precatorius</i>	Hideki <i>et al.</i> , 2004
	Lfcin-B peptide	Lactoferrin-B protein	Yoo <i>et al.</i> , 1997

Purification of peptides and characterization

Proteins and their constituent amino acids are present abundantly in many food sources. The separation of bioactive peptides from enzymatic hydrolysates provides scope to create functional foods that can slow disease progressions. Isolation and identification of proteins or peptides of interest for bioactive functional roles is thus a very important step. Peptides are known to be important biological modulators and proper analytical separation and purification techniques can alone identify specific amino acid sequences for reproduction and synthesis. Isolation and separation of protein/peptide of interest from a pool of proteins becomes a challenging task, because many proteins/peptides can possess closely related physicochemical properties (Pouliot *et al.*, 2006). Fractionation and purification techniques are normally performed based on where and for what purpose the final product/molecule is rendered applicable. For purification of food components expensive methods such as chromatography are used to achieve purity for industrial applications.

Various preparative chromatographic methods have been used to purify proteins and peptides, including, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography and size exclusion chromatography. Out of several separation

techniques available High Pressure Liquid Chromatography (HPLC) happens to be the most widely used method for analytic as well as preparative separations of proteins and peptides. Reverse phase HPLC is extensively employed with (or without) pairing to ion-exchange chromatography for efficient purification of single peptides from a pool (Byun *et al.*, 2001, Stepnowski *et al.*, 2005, Kannan *et al.*, 2008). Conventional UV and fluorescence detectors enable detection of proteins and peptides of interest but, structural characterization of purified peptides can be achieved by amino acid analysis, SDS–electrophoresis and mass spectrometry (MS). Furthermore, MS analysis can include matrix-assisted laser desorption/ionization (MALDI) to enable precise structural information and characterization.

Ion-exchange chromatography is used to separate molecules based on their charge where the retention time in the solid matrix (column) also depends on the electrostatic interactions. The isoelectric point (pI) is the most important property of proteins and their products, which is utilized in effective purification techniques. Various peptides were subjected to ion exchange chromatography and other effective purification techniques from their native proteins, for example, Lactoferricin was isolated from Lactoferrin (Dyonisius et al, 1997, Recio *et al.*, 2000) and Luffacylin isolated from a vegetable (*Luffa acutangula*) seed protein (Parkash et al, 2002).

Size exclusion chromatography is another technology that can be used to separate relatively large peptides (>1kDa). The column gel matrix for this method of separation consists of a definite pore size for the molecules to pass or retain based on their size. Smaller molecules diffuse faster than their larger counterparts and the differences in the retention time allows for their separation. Generally, size exclusion when coupled with ion exchange and HPLC can produce better separation as the molecules are separated by size as well as the net charge which determines their distinct retention time (Prakash *et al.*, 2002).

Nisin, a peptide from bacteria classified as bacteriosin, was identified and purified using a combination of chromatographic methods. The researchers established a two-step process where the sample were adsorbed on a hydrophobic resin followed by ion (cation) exchange resin which enabled a 100% recovery of the compound (Stoffels *et al.*, 1993). Using sepharose cation-exchange gel elution with NaCl and by HPLC technique using low pressure were studied by Uteng *et al.* (2002) which resulted in 90% recovery of the bacterial peptide. Hence, characterization of peptides can be accomplished using single or combinations of chromatographic. However, efficiency of the purification process requires various trials of optimizing the technique and hence substantiates as a challenging action. Recent automation and sophistication of instruments has aided in quicker and convenient procedures for protein separations.

High pressure liquid chromatography or Reverse Phase-HPLC has become extremely popular for protein separation and purification techniques to obtain purified peptides. In reversed phase, separation is achieved through partition and adsorption where the stationary phase is non-polar (or less polar than in the mobile phase). The analytes are retained on the column until a polar solvent (in the case of a mobile-phase gradient) is used to elute them. Large peptides (>4,000 Da) are analyzed with columns with greater pore diameter (300Å) while the smaller molecular pore sizes (60-100Å) are preferred for separation of smaller peptides. The physicochemical characteristics of peptides, hydrophobic or hydrophilic, influence the choice of stationary phase used. Acetonitrile is the most widely used organic solvent for peptides since it has high transparency during UV detection between 200 and 220 nm. Ion-pairing reagents including organic acids, have been used to increase efficiency in separation and to yield pure peptides (Piot *et al.*, 1992, Dionysius and Milne 1997, Hoek *et al.*, 1997, Kannan *et al.*, 2010).

Initial identification followed by purification using RP-HPLC separation technique with Mass spectrometry and Matrix Assisted Laser Desorption Ionization-Time of Flight were conducted for peptides obtained from food products with bioactivities and antimicrobial properties (Shimazaki *et al.*, 1998, Leonil *et al.*, 2000). The following table (2.4) illustrates the types of HPLC used for separation of food peptides, their separation mechanism and characteristics of separation.

Table 2.4. HPLC methods involved with food peptides (Nollet, 2004).

HPLC type	Separation mechanism	Characteristics
RP-HPLC	Hydrophobic interaction of the peptides with the stationary phase	High resolution High speed Standard separation procedures Prediction of retention times Good detection and reproducibility Quantitative recovery using volatile buffers Moderate capacity in analytical columns
IE-HPLC	Ionic interaction of peptides with anion exchangers or cation exchangers as stationary phase	Moderate resolution High speed High capacity Prefractionation of peptides by charge Wide range of pH
SE-HPLC	Size exclusion of peptides on hydrophilic polymers as stationary phase	Moderate or low resolution High speed Very high capacity Desalting Good prefractionation by size Molecular weight determination Presence of nonspecific interactions

Characterization of proteins and peptides using proteomic tools

Characterization protocols have been made simpler, quicker and reliable with the arrival of modern proteomic techniques that aid in precise and quick identification and detection of structural components of a peptides within a complex as well as pure mixture. Several proteomic tools were developed based on several factors that are involved in characterizing a compound's

purity and to ascertain its functionality. Coupling of chromatography and spectrometry in tandem has enhanced separation, improved mass determining capabilities, decreased process time and increased sensitivities. Mass spectrometer and its accessory coupling instruments are the most popular tools available for studying proteins.

Mass spectrometry (MS)

It is an analytical technique for determination of the elemental composition and chemical structure of molecules such as peptides. The principle is to ionize chemical compounds to generate charged molecules or their fragments and measure their mass-to-charge ratios (Horn *et al.*, 2000). In an MS protocol the sample is impacted with electron beam to ionize the compounds. The resultant charged particles are subjected to an electric field where the separation of molecules occurs based on mass-to-charge ratio which are measured to identify the individual compounds. There are two methods for ionization during MS, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The latter is used for thermolabile and non-volatile compounds with high molecular mass. MALDI is used successfully in biochemical studies for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides (Hillenkamp, 1991). The accuracy of the compounds mass is based on analyzer type and performance. However, most advanced instruments will be capable of measuring masses to within 0.01% of the molecular mass of the sample, up to 40,000 Da.

The MALDI technique based ionizing the samples by bombarding with a laser light is the standard procedure before a low concentration of the sample is pre-mixed with a highly absorbing matrix compound for the most consistent outcomes. Sputtering of analyte and matrix ions from the surface of the mixture occurs when the laser energy is transformed into excitation energy. Since this energy transfer is efficient the analyte molecules are spared from

decomposition by avoiding excessive direct energy. The time-of-flight (TOF) analyzer attached to the MALDI uses an electric field to accelerate the ions through the same electric potential, and measures the time they take to reach the detector. The compounds are identified based on their velocity to reach the detector which depends exclusively on their mass.

Tandem mass spectrometry (MS-MS) is the most advanced screening method for structural information about a compound. It uses fragmentation of specific sample ions inside the mass spectrometer to identify them and the information is pieced together to generate structural evidence of the intact molecule. A tandem mass spectrometer is a specialized instrument that displays results in the form of a mass spectrum, which is a graph that shows each specific molecule by weight (mass) and how much of each molecule is present. It can sort biochemically important molecules of similar weight in a sample and can detect specific compounds in complex mixtures based on their characteristic fragmentation patterns. Peptides fragment in a reasonably well-documented manner where the protonated molecules fragment along the peptide backbone and also show some side-chain fragmentation with certain instruments. The three different bonds that can fragment along the amino acid backbone are the NH-CH, CH-CO, and CO-NH (most common), which give rise to the “b” and/or the “y” ions. Each amino acid residue is indicated by the mass difference between two adjacent “b” ions, or “y” ions.

Amino acid sequences of peptides

The exact sequence and the number of amino acids that constitute proteins/peptides is determined by the n-terminal amino acid sequence. Proteins are probed for the occurrences of functional di- or tri-peptides within their structures. This is to identify critical areas that impart significant functional characteristics to the protein. Further, these findings provide exact amino acid composition of functional peptides for *in vitro* synthesis. Di-deoxy method is the standard

for N-terminal amino acid sequencing and these studies have been done several times when peptides are involved. Alcalase treatment of soybean proteins formed low molecular weight peptides which are isolated and identified to be effective against hypertension. A sequence of 9 amino acids obtained from hydrolysis of rice albumin protein was found to promote immuno-stimulation. Several peptides ranging in amino acids of about 5-10 have been successfully isolated and proven beneficial against disease characteristics. Determination of the correct sequence and number of amino acids that constitute proteins/peptides is by the N-terminal amino acid sequence of proteins and peptides.

The activity of biologically functional peptides is due to their amino acid composition and sequence. For example, two peptides purified from canola protein hydrolysate were shown to inhibit ACE-I activity after purification. The amino acid analysis of the peptides showed 45% content of aromatic amino acids in comparison to 8.5% of the hydrolysate (Wu et al, 2008). Many peptides are known to have multifunctional properties as the size of their active sequences may vary from two to twenty amino acid residues (Meisel and FitzGerald, 2003). For example, in β -casein amino acid sequence the peptides 60-70 show immune-stimulatory, opioid and ACE-I inhibitory activities. Hence, these sequences are classified as strategic zones and are protected from proteolysis due to proline residues and high hydrophobicity (Migliore-Samour and Jolles, 1988; Meisel, 1998). There are several examples of multi-functional peptides from various sources including milk and rice (Vogel, 2012, Kannan *et al.*, 2012). The α s1-casein fraction 194-199 showing immune-modulatory and ACE-I inhibitory activity (Korhonen and Pihlanto, 2003). The C-terminal moiety of κ -casein, residues 106–169, cleaved by chymosin from Caseinomacropetide (CMP) during cheese making was reported to have diverse nutritional and biological significances due to unique amino acid composition. Researchers have suggested

CMP as a protein source for the treatment of Phenylketonuria, a hereditary disorder in which aromatic amino acids cannot be metabolized, since CMP does not contain the aromatic amino acid residues (Leonil et al, 2000).

Proteins can thus be probed for the occurrences of functional di- or tri-peptides within their structures to identify critical areas that impart significant functional characteristics to the protein. The findings can also provide amino acid composition of functional peptides and the precise sequence information can help peptides to be synthesized in large scale for ingredient formulation as a nutraceutical agent.

Biological activities of soybean peptides

Soy peptides have been studied for disease resistance and also for biological activity against spread of certain non-pathological diseases. Research has found out that hydrophobic soy peptides with anticancer activity have arrested cancer cell cycle progression in the in vivo studies. The mouse monocyte macrophage cell line P388D1 was incubated with the soy peptides. The peptides arrested the cell growth at the G2/M phase of the cell cycle. The cells were stained with propidium iodide, and DNA analysis of each phase was done using the Fluorescent Activated Cell Sorter (FACS) equipment (Kim *et al.*, 2006). Numerous peptides with various bioactive functions have been identified in soy. Lunasin is one such peptide from soy which is 43 amino acid residues long and has been shown to be chemo-preventive in mammalian cells and in a skin cancer mouse model against oncogenes and chemical carcinogens. (Jeong H. J. *et al.*, 2003). In the Bio-pep database (Dziuba J. *et al.*, 2003) more than 1500 different bioactive peptides like angiotensin converting enzyme (ACE-I) inhibitors and dipeptidyl peptidase IV inhibitors have been listed. Some peptides are with opioid agonistic and antagonistic, antioxidative, anticancer, anti-hypertensive, and immuno-modulatory actions. The present

research is to develop peptides from soy with biological activity against hypertension and cancer cells.

Emphasizing the amino acid roles in cancer progression, and the lack of studies on involvement of protein hydrolysates and peptides in ACE-I (potential biochemical agent for inducing hypertension) and multiple-site cancer (colon, lung, liver, breast, prostate and blood cancer cell lines) proliferation inhibition, we propose to investigate roles of peptides extracted from soybeans (an established source of flavonoids and other compounds responsible for anti-proliferative effects). Literature information on soybean proteins/peptides on control of cancer proliferation has been mostly involving soy isoflavones and naturally occurring peptides that have anti breast-cancerous effects, or other bioactive functions. However, nothing has been done using high oleic acid soybeans with comparatively higher amounts of protein as well as methionine and cystine to prepare medium sized synthesizable bioactive peptides. Research in genetically engineered high oleic acid soybean has a great future, since there is production of higher quality vegetable oil and utilization of the byproduct from the oil industry in developing certain nutraceuticals that can be the future of disease prevention and control. Knowing the amino acid sequences of these peptides would enable the industry to produce synthetic peptides with equivalent bioactivity.

Studies with soybean components will emphasize that the incidence of cancers are much lower in Asian countries where soy is used as chief ingredient in the population's staple diet. Several technological options available in the literature are on the production, preparation, gastrointestinal resistance evaluation, identification of specific bioactivities, and separation and purification strategies for food derived peptides. Depending upon the nature of the bioactivity and the target site it is critical to consider all the options available before conditions can be

established for the production of bioactive peptides. With such an understanding peptides derived from soybean meals have been prepared and evaluated for anti-ACE-I and anticancer activities. In the following chapters the ways adopted for preparation and characterization biologically active soybean peptides have been documented.

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CHAPTER 3

Protein content and amino acid profiling of 44 soybean lines developed in Arkansas

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Abstract

Soybeans are cultivated in the United States chiefly for cooking oil, while the residue after oil extraction (soybean meal) is mostly used in animal feed formulations. High protein content in the defatted soybean meals led to its extraction and application in food products due to its good quality. However, soy protein is naturally deficient in sulfur-containing amino acids, methionine and cysteine. But, in recent years, plant breeding techniques have been used to enhance the protein and fatty acid quality in soybeans. In this study, forty four selected soybean lines were evaluated for their moisture and protein contents and amino acid composition. The moisture was determined by AACC air oven method, protein content by an automated Kjeldahl method and the amino acid profiling was conducted based on AOAC official method. The moisture percentage ranged between 5.2 and 12.9 % among the lines while the protein content on dry basis ranged between 40 and 53 %. The amino acid analysis showed high methionine content in the R05-4494 and R05-5491 with 'high protein and fatty acid' as yield attributes. High cysteine content was found in one of the high oleic acid soybean line, CRR05-188, which is comparable to that in the check lines, UA-4805 and 5601-T. The results indicated that fatty acid associated attributes in the soybean lines did not influence the protein content. This study will promote newly developed specialty soybean lines with high oleic acid and high protein content that can add value to the crop and provide nutritious ingredients for food product manufacturers.

Introduction

Soybean is an excellent source of protein and oil and is the second major crop grown in the United States. The USA is the largest producer and exporter of soy in the world and the forecast for 2014 production is approximately 3.3 billion bushels (~27 kg/bushel) according to the Crop Production Report by the United States Department of Agriculture (2012). The American Soybean Association and the regional soybean boards have a major role in producing better quality soybeans using genetic engineering and plant breeding techniques by improving various attributes including enhanced yields, pest and disease resistance, lipid quality and quantity, and protein content and quality (Cober and Voldenga, 2000, Cahoon, 2003). Soybeans are primarily grown for the edible oil which is separated by various extraction methods, resulting in a leftover residue called soybean meal. Soybean meal is a major ingredient in animal feed formulations as a source of complete protein. It is also a chief source of high-quality plant-based protein in human diet. Hydrolysates prepared from the soybean proteins are in great demand as ingredients for food applications as well as in protein supplements that provide nutritional and health benefits (Reynolds *et al.*, 2006, Genovese *et al.*, 2007, de Moura *et al.*, 2008). Improvements in soybean processing and functional characteristics have diversified the ever-increasing demand for soy protein ingredients (Uzzan and Labuza, 2004).

New soybean lines are produced with higher yields, higher protein content, and recently with higher amounts (up to 80 %) of oleic acid in their lipid composition (Clemente and Cahoon, 2009). A higher percentage of monounsaturated fatty acids such as oleic acid is preferred over polyunsaturated fatty acids which have lower oxidative stability (O'Keefe *et al.*, 1993, Warner and Knowlton, 1997). Variations in the plant genes are the cause for differences, not only in the oil content but also in the concentration of crude protein, possibly even in the amino acid

composition (Takagi and Rehman, 1996, Mazur *et al.*, 1999, Monteros *et al.*, 2008). Researchers have found an increase in lysine in the hybridized high oleic acid soybean seeds compared to the parent lines (Dixon, 1996, Health Canada, 2000). The amino acid composition of the seed protein depends on the storage protein content, nitrogen supply during the growth phase, and asparagine levels in the embryonic stage of the plant (Sebastia *et al.*, 2005). There has not been much research in evaluating the essential amino acid content, specifically sulfur-containing amino acids in the soy protein from high oleic acid soybean seeds. Oil-extracted soybean meals contain approximately 1 % residual oil and have 48 % crude protein with all the essential amino acids required for human health except for the sulfur amino acid methionine (Krishnan, 2005).

This is the first time that amino acid analysis of soybean lines with varying oleic acid composition has been studied. The 44 soybean lines used in this study were bred for attributes including yield, protein content, low linoleic acid, high oleic acid content, high yield and high protein. The major objectives of this study were to analyze the protein and its amino acid composition among 44 soybean lines.

Materials and Methods

The seeds of 44 soybean lines (R05-4509, R95-1705 (non-GMO), R05-4476, R05-4487, R05-4473, R05-4507, R05-4492, Satellite, R05-4494, S03-543CR, N98-4445, R05-5491, R05-5340, R05-4457, R05-4478, R05-4505, R05-5362, Osage, S01-9265, UA-4805, 5601-T, R05-5351, S04-4729RR, TN01-235, S04-3835 RR, R05-5510, Satellite, N98-4445, Kristine, 5002-T, IA-3017, TN-5123, R05-4481, R05-5342, R05-5494, CRR05-188, V01-1693, V01-6338, V01-1702, S01-9364, Ozark, IA-2064, R05-5358, KS-5007) from two Arkansas Agricultural Research Stations (ARS) based in Fayetteville (FAY) and Stuttgart (STU) were provided by Dr. Pengyin Chen, Plant breeder and Professor, Department of Crop Soil and Environmental

Sciences, University of Arkansas. Kjeltex 2200 auto-distillation unit (Foss, Eden Prairie, MN) was used to determine the protein content in the flour. Rotovapor (Buchi, Flawil, Switzerland) was used for vacuum distillation and Beckman HPLC system (Fullerton, CA) was used for quantitative amino acid analysis. An Ika mill (Ika-Werke, Staufen, Germany) was used for grinding the samples. All chemicals, solvents, and reagents with highest purity were purchased from Sigma (St. Louis, MO, USA).

Moisture and Protein Content Determination.

The seeds were ground, passed through a 60-mesh sieve, and the flour was collected, bagged and stored at 5 °C. The moisture percentage of the flour was calculated based on the AACC official method (2000). The soybean flour samples were weighed in aluminum pans and dried at 135 °C for 3 h. The moisture percentage was calculated as the ratio between the moisture lost from the sample and the actual weight of the sample before drying. This was done in triplicate for all the 44 seed samples. Protein content of the soybeans was performed using the Kjeldahl method (AOAC, 1997). The soybean flour samples were weighed in digestion tubes and digested for 1 h at 420 °C after adding the Kjeldahl tablets and 10 mL sulfuric acid. The samples were distilled and titrated against 0.1 N HCl in the automated Kjeldahl distillation unit. The protein percentage was calculated with a conversion factor of 6.25 for nitrogen. The moisture percentage was used to calculate the protein content by dry weight for all the samples. All analyses were conducted in triplicate.

Determination of Amino acid Composition.

The amino acid analysis of the flour from the 44 lines was conducted using the AOAC method (1997). The approximate weight of each soybean meal test sample for amino acid analysis was calculated by the formula: $W_s = 1000/N_s$; where W_s is the weight of the sample in

milligrams, and N_s is the nitrogen content (%) in each sample. Performic acid solution was prepared and kept at room temperature for 30 min and cooled in an ice bath for 15 min before adding to the samples. All flour samples were weighed into 250-mL Erlenmeyer flasks and cooled in an ice bath. Five milliliters of performic acid was added to each flask with the sample, stirred for 15 min, and all flasks were kept in an ice bath for 16 h for oxidation. After oxidation, the performic acid was decomposed by adding 0.84 g of sodium metabisulfite to each sample flask under a fume hood and stirring for 10 min. The oxidized products were hydrolyzed in 6 M HCl-phenol solution for 24 h at temperatures between 110 and 120 °C. The hydrolyzed sample solution was cooled to room temperature, and 20 mL norleucine solution was added as HPLC elution standard. The solutions were evaporated using a rotary evaporator under vacuum (until 5–10 mL remained), diluted with sodium citrate buffer and the pH adjusted to 2.2. The volume of the hydrolyzed sample solution was made up to 50 mL with the buffer solution and stored at 5 °C in polyethylene bottles. The solutions were injected into a C18 ion exchange column (heated to 70 °C) using an auto-sampler attached to the HPLC system, and the amino acids were detected based on the absorbance measured at 254 nm. Eluent buffer solutions, procured from Pickering Laboratories (Mountain View, CA, USA), containing sodium citrate and hydrochloric acid at varying pH (3.2, 4.2 and 6.4) were used. The elution times of each amino acid on the column were compared to an amino acid standard and the amount of each amino acid was calculated in mg/g based on the peak area.

Statistical analysis:

The JMP software from SAS institute (Cary, NC) was used for the statistical analyses – Student's t test, analysis of variance, means and standard deviations (P value <0.05). The data were collected in triplicate for all the experiments.

Results and Discussion

Moisture and Protein Content.

The moisture percentage in the flour among the 44 soybean lines ranged between 5.2 ± 0.0 and 12.9 ± 0.1 % (Table 3.1). The moisture values varied due to the difference in the agronomic growing conditions of the lines, moisture content at harvest and the processing conditions of the 44 lines. Nevertheless, the moisture content did not have a significant impact on the protein content in the seeds.

The protein percentage among all 44 lines ranged between 40.8 ± 0.2 and 53.5 ± 0.1 % approximately by dry weight. The differences in the protein content among the soybean lines could be due to the genetic variation among the soybean lines. There was a statistically significant difference in the protein content of the soybean lines as shown in Table 3.1. Both Fayetteville and Stuttgart Agricultural research stations produced high protein lines. The variation in the protein content among the lines could be attributed to the dissimilarities in the soils of the two regions. R05-4509 (STU), R05-4476 (STU), R05-4487 (STU), R05-4473 (STU), and R95- 1705 had the highest protein yields which were not significantly different from each other according to the Student's t test ($P > 0.05$) while R05-4509 (STU) showed the highest protein content of 53.5 ± 0.1 % among all the lines.

The highest protein content on dry basis (d. b.), 53.5 ± 0.1 % was from high protein line and fatty acid line, R05-4509, which was not significantly different (statistically) from that of the high yielding line, R95-1705 with 52.7 ± 0.4 % protein. The protein content of R05-4509 (STU), R95-1705 R05-4476 (STU), R05-4487 (STU), and R05-4473 (STU) was much higher than normal and those with high oleic acid, S03- 543CR and N98- 4445A (Fay), are also found to have significantly high protein content. Lines grown for the high yield and high protein attributes

had higher protein content than the high oleic acid lines which is consistent with previous studies (Cober and Voldenga, 2000). Two other high oleic acid lines, CRR05-188 and TN-5123 from the Fayetteville ARS stations, had 45.2 and 44.5 % protein which are not statistically different from each other and similar to the ‘check’ lines—N98-4445A foundation and 5002-T. The ‘check’ soybean lines are used as positive control for comparison of an attribute (high yield, high protein or high oleic acid, etc.) among the new breeds of soybean seeds developed. ‘Foundation’ lines are those developed by the breeder (copyrighted) for distribution among the growers. The lowest amount of protein on dry basis was found in the KS-5007 low linolenic acid soybean which is 40.8%.

Amino Acid Content.

The amino acid analysis of the 44 soybean lines showed variability in protein composition, while the presence of high oleic acid content did not provide a wide variation. Hence, other components, including isoflavones and oligosaccharides in the seed, could utilize the available carbon skeletons during development. A positive correlation between protein, lipid, sugars and isoflavones during the soy seed growth and maturity have been observed in previous studies (Kim *et al.*, 2006). The essential amino acid composition of the seed protein in 44 lines is given in Table 3.2. The amino acid composition among the high oleic acid soybeans lines did not differ significantly (P value <0.05, data not included). High methionine and cysteine content were observed in lines: R05-4494, R05-5491, 5002 T, Kristine, R05-5362 and R05-5352, which were 53.7 ± 1.4 , 43.5 ± 0.1 , 41.8 ± 4.8 , 41.0 ± 0.8 , 35.2 ± 3.6 and 39.9 ± 0.4 mg/g, respectively. Table 3.3 shows the list of soybean lines which had the highest amounts of methionine content which can be sources of complete protein. This could be due to the nitrogen assimilation during the seed development which determines the amino acid composition of the seeds (Sebastia *et al.*,

2005). The methionine content in the six soybean lines (addressed above) are significantly higher in comparison to egg (34 mg/g) and milk (20 mg/g) proteins (Table 3.4). Cysteine is the other sulfur containing amino acid which is non-essential to humans but is required for the maintenance of protein structure and function. Table 3.3 shows the soybean lines which had the highest cysteine content: R05-4505— 35.5 ± 0.3 mg/g, S01- 9265 — 30.8 ± 4.8 mg/g, Satellite STU 23.7 ± 1.5 mg/g and CRR05-188— 25.5 ± 3.3 mg/g which is a high oleic acid line. These values are higher than those found in milk protein (8 mg/g) and are equal or higher in comparison to egg protein (24 mg/g).

The CRR05-188 is the only high oleic soybean line that showed higher cysteine content, although its seed protein content is lower in comparison to S03-543CR and N98- 4445A (high oleic acid lines). This indicates that sulfur amino acid content in soybeans is not related to the amount of total seed protein. The soybean line S03-543CR showed the highest amounts of essential amino acids—threonine, valine, isoleucine and leucine among all the lines tested irrespective of their traits. It also showed significantly high amounts of lysine amino acid when compared with the 44 soybean lines. This shows that soybean lines bred for high oleic acid can also provide substantial essential amino acid content along with high protein. The protein content in the TN-5123 high oleic acid soybean line was not significantly different in comparison to CRR05-188, but the essential amino acid content was lower in comparison to other high oleic acid lines. Hence, other factors, including soil environment and growth conditions, affect the protein formation during seed development.

Among all the soybean lines, the compositions of glutamine, asparagine and lysine amino acids are highest in that order, respectively (Fig. 1). Glutamine is essential for gut health while asparagine maintains the integrity of the nervous system (Newburg and Fillios, 1982, Tuohy *et*

al., 2003). Lysine is known to be an essential amino acid which helps in serotonin regulation and has a moderating effect on blood pressure and the incidence of stroke (Tuohy *et al.*, 2003, Smriga *et al.*, 2002). The amino acid analysis procedure from AOAC official methods (994.12) was selected to quantify the sulfur-containing amino acids methionine and cysteine. However, quantification of aromatic amino acids, tryptophan and tyrosine, was affected during hydrolysis and oxidation and these were not detected during the ion-exchange liquid chromatography.

All 44 soybean lines showed high amounts of lysine which agrees with the accepted notion that legume seeds are rich in this amino acid (Iqbal *et al.*, 2006). While glutamine and asparagine were non-essential amino acids, supplementation of lysine is essential to humans since it is not synthesized in the body. Lysine plays an important role in transamination reactions and is utilized to produce vital proteins, including elastin and collagen (Eyre *et al.*, 1984, Reiser *et al.*, 1992, Akagawa and Suyama, 2001). The comparison of essential amino acids (range) among the tested 44 soybeans and other protein sources including eggs and milk are given in Table 3.4.

Researchers have engineered quality traits through plant breeding to enhance the sulfur amino acid content, even though natural mutations in soybeans have also expressed these beneficial effects. The genetic alterations have been tested specific to the trait loci that trigger the formation of methionine and cysteine during the seed growth (Fujiwara *et al.*, 1992, Krishnan, 2005). Soil sulfur and nitrogen content during the growth of the soybeans also affects the methionine and cysteine content in the soybean protein (Krishnan *et al.*, 2005). Other genetic attributes like high protein, high monounsaturated fatty acid content, and higher yields or disease resistance may contribute to enhanced sulfur amino acids, but this is inconclusive.

Conclusion

The protein contents of soybean lines R05-4509, R95-1705, R05-4476 and R05-4487 were higher among all the lines tested. Soybean lines with ‘high oleic acid’ (S03- 543CR and N98-4445A) were also found to have substantially enhanced protein content (approximately 48 % d. b.). Lines grown for the ‘high protein and fatty acid’ attribute had 47–52 % (d. b.) protein content which was comparable to that in the ‘high oleic acid’ lines. Amino acid analysis showed a significantly higher methionine levels (P value <0.05) in soybean lines recognized for protein and fatty acid content (R05-4494 and R05-4478). Both methionine and cysteine contents were elevated in the soybean lines attributed for protein and fatty acid.

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Table 3.1. Seed moisture and protein content of selected 44 Aransas-grown soybean lines.

Cultivar ¹	Moisture %	Protein % (dry basis)	Yield Attributes ²
R05-4509 (STU)	11.9 + 0.0def	53.5 + 0.1a	High protein and fatty acid
R05-4473 (STU)	13.1 + 0.1def	52.1 + 0.2b	High protein and fatty acid
R05-4507 (STU)	12.0 + 0.0kl	50.1 + 0.1c	High protein and fatty acid
R05-4492 (STU)	11.5 + 0.0efg	49.6 + 0.6cd	High protein and fatty acid
R05-4494 (STU)	11.8 + 0.3efg	48.6 + 0.0def	High protein and fatty acid
R05-4457 (STU)	5.3 + 0.1rs	47.4 + 0.2fghij	High protein and fatty acid
R05-4478 (STU)	6.1 + 0.1pqrs	47.4 + 0.1fghij	High protein and fatty acid
R05-4505 (STU)	11.4 + 0.1a	47.3 + 0.1ghijk	High protein and fatty acid
R05-4481 (STU)	12.6 + 0.1efg	45.2 + 0.3nop	High protein and fatty acid
R95-1705 (FAY)	6.4 + 0.0qrs	52.7 + 0.4ab	High yield
Osage (FAY)	6.1 + 0.1pq	46.7 + 0.3ijkl	High yield
S01-9265 (FAY)	9.4 + 0.2kl	46.5 + 0.2ijklm	Low saturated fat
Satellite (FAY)	6.7 + 0.2opq	45.7 + 0.3lmnop	Low saturated fat
V01-6338 (FAY)	5.7 + 0.1qrs	43.7 + 0.2qr	Low saturated fat
UA-4805 (STU)	11.6 + 0.0bcde	46.4 + 0.6ijklmn	Check
5601-T (STU)	11.2 + 0.0bcde	46.4 + 0.2jklmn	Check
5002-T (STU)	11.7 + 0.0efghi	45.2 + 0.3nop	Check
S03-543CR (FAY)	6.2 + 0.2pqr	48.4 + 0.5defg	High oleic acid
N98-4445A (FAY)	8.4 + 0.1m	48.1 + 0.4efg	High oleic acid
TN-5123 (FAY)	7.6 + 0.2mno	45.2 + 0.3nop	High oleic acid
CRR05-188 (FAY)	6.4 + 0.0pq	44.5 + 0.4pq	High oleic acid
S04-4729RR (FAY)	7.9 + 0.3bcde	46.1 + 0.1klmno	Low linolenic acid
TN01-235 (FAY)	8.2 + 0.3m	46.0 + 0.6lmno	Low linolenic acid
S04-3835 RR (FAY)	8.1 + 0.1mn	46.0 + 0.4lmno	Low linolenic acid
Kristine (FAY)	6.2 + 0.0abcd	45.4 + 0.8mnop	Low linolenic acid
IA-3017 (FAY)	7.9 + 0.2hijk	45.2 + 0.2nop	Low linolenic acid
V01-1693 (FAY)	9.7 + 0.1mn	43.8 + 1.4qr	Low linolenic acid
V01-1702 (FAY)	10.3 + 0.0mn	43.7 + 0.6qr	Low linolenic acid
S01-9364 (FAY)	7.1 + 0.1nop	43.5 + 0.8qr	Low linolenic acid
IA-2064 (FAY)	5.2 + 0.0s	42.8 + 0.2r	Low linolenic acid
KS-5007 (FAY)	10.5 + 0.2l	40.8 + 0.2s	Low linolenic acid
Ozark (FAY)	8.3 + 0.0m	43.2 + 0.3r	Conventional
R05-4476 (STU)	11.6 + 0.0jkl	52.6 + 0.1ab	Fatty acid

Table 3.1. Seed moisture and protein content of selected 44 Aransas-grown soybean lines (cont.).

Cultivar ¹	Moisture %	Protein % (dry basis)	Yield Attributes ²
R05-4487 (STU)	11.9 + 0.0def	52.3 + 0.2ab	Fatty acid
Satellite(STU)	12.0 + 0.0defg	48.9 + 0.1de	Fatty acid
R05-5491 (STU)	10.4 + 0.1fghij	47.6 + 0.2efghi	Fatty acid
R05-5340 (STU)	10.7 + 0.4abc	47.4 + 0.5fghij	Fatty acid
R05-5362 (STU)	10.8 + 0.2ab	46.8 + 0.2hijkl	Fatty acid
R05-5351 (STU)	11.5 + 2.1pqr	46.3 + 0.8jklmno	Fatty acid
R05-5510 (STU)	9.9 + 0.1cde	45.7 + 0.4lmnop	Fatty acid
R05-5342 (STU)	12.9 + 0.1ijk	45.1 + 0.4op	Fatty acid
R05-5494 (STU)	11.8 + 0.1ghij	44.6 + 0.3pq	Fatty acid
R05-5358 (STU)	9.7 + 0.1efgh	41.0 + 0.2s	Fatty acid

Values given are averages of three replications \pm standard deviation and those not connected by same letter in each column are significantly different ($P < 0.05$). ¹STU is abbreviated for Stuttgart and FAY is abbreviated for Fayetteville. ²Yield attributes are the traits specific to the breed of the developed lines. (Rayaprolu *et al.*, 2015).

Table 3.2 (a-h). Essential amino acid composition (mg/g protein) in 44 soybean lines grown in the state of Arkansas.

(a)

Amino acid	R05-4509	R95-1705	R05-4476	R05-4487	R05-4473	R05-4507
Cysteine	15.2±0.0 ^{ab}	14.8±0.4 ^a	15.1±0.5 ^{ab}	14.5±0.7 ^a	13.4±0.8 ^a	14.6±0.3 ^a
Threonine	44.1±0.7 ^{mn}	41.8±0.6 ^l	43.7±0.7 ^m	42.2±0.5 ^{lm}	36.8±0.5 ^k	41.6±0.6 ^l
Valine	31.4±0.9 ^h	40.7±0.5 ^l	35.5±0.8 ^{ij}	29.5±0.6 ^g	23.1±0.6 ^{cd}	38.2±0.5 ^k
Isoleucine	32.7±0.8 ^{jk}	40.2±0.3 ^p	37.1±0.8 ⁿ	32.0±0.5 ^{jk}	22.3±0.5 ^e	36.2±0.4 ^m
Leucine	67.4±0.7 ^k	68.9±0.5 ^l	67.4±0.6 ^k	64.2±0.6 ⁱ	55.6±0.5 ^{de}	64.5±0.3 ⁱ
Lysine	109.0±1.0 ^q	113.5±0.9 ^s	112.0±0.9 ^{rs}	107.3±1.3 ^p	101.6±0.9 ^m	109.5±0.7 ^q
Methionine	22.3±1.2 ^f	24.3±0.9 ^g	23.5±1.0 ^g	20.6±1.1 ^{ef}	19.1±0.7 ^e	21.4±0.9 ^f
Phenylalanine	40.5±0.9 ^j	46.7±0.6 ^m	45.1±0.8 ^{lm}	46.0±0.9 ^m	41.4±0.6 ^k	45.4±0.4 ^{lm}
Histidine	53.8±0.6 ^{jk}	56.3±0.4 ^l	54.7±0.6 ^k	51.6±0.5 ⁱ	44.9±0.5 ^g	52.7±0.4 ^j

(b)

Amino acid	R05-4492	Sat (STU)	R05-4494	S03-543CR	N98-4445A	R05-5491
Cysteine	16.5±0.0 ^b	23.7±1.5 ^e	15.9±0.1 ^{ab}	14.2±0.5 ^a	14.9±0.3 ^a	15.5±0.6 ^{ab}
Threonine	21.0±0.0 ^{ef}	21.8±0.0 ^{ef}	20.0±0.1 ^{de}	45.2±0.7 ⁿ	18.5±0.6 ^d	21.3±0.4 ^{ef}
Valine	29.2±0.1 ^g	22.7±0.1 ^c	25.6±0.6 ^e	42.1±0.5 ^m	27.9±0.4 ^f	33.8±1.4 ⁱ
Isoleucine	24.3±0.1 ^f	22.0±0.0 ^e	22.5±0.0 ^e	39.2±0.6 ^o	25.9±0.3 ^f	31.6±1.7 ^j
Leucine	66.7±0.4 ^{jk}	57.8±0.4 ^{ef}	68.3±0.2 ^{kl}	69.1±0.5 ^l	51.3±0.5 ^b	71.2±3.9 ^m
Lysine	109.7±1.5 ^q	60.7±0.3 ^g	98.1±6.5 ^k	123.8±0.8 ^u	53.4±0.7 ^{de}	117.2±7.0 ^t
Methionine	26.4±0.3 ^h	17.1±0.1 ^d	53.7±1.4 ^p	24.3±1.2 ^g	14.2±0.0 ^{bc}	43.5±0.1 ^o
Phenylalanine	47.1±0.1 ⁿ	35.4±0.1 ^{fg}	31.1±2.7 ^d	48.0±0.4 ^o	30.1±0.2 ^c	46.1±3.0 ^m
Histidine	52.5±0.1 ^j	33.6±0.0 ^{cd}	49.3±0.4 ^h	56.2±0.5 ^l	30.9±0.2 ^b	56.6±2.5 ^l

(c)

Amino acid	R05-5340	R05-4457	R05-4478	R05-4505	R05-5362	Osage
Cysteine	14.6±0.6 ^a	21.3±2.3 ^{cd}	15.3±0.1 ^a	35.1±0.3 ^h	14.9±0.2 ^a	14.7±0.4 ^a
Threonine	19.4±0.3 ^d	21.6±0.0 ^{ef}	19.9±0.2 ^d	21.2±0.0 ^{ef}	18.6±0.8 ^d	41.4±0.7 ^l
Valine	35.0±1.0 ^{ij}	23.8±0.0 ^{cd}	26.5±0.4 ^{ef}	20.2±0.1 ^{bc}	36.4±0.8 ^j	24.7±0.9 ^d
Isoleucine	31.8±0.3 ^j	23.6±0.0 ^{ef}	22.9±0.0 ^{ef}	19.6±0.1 ^d	31.3±0.2 ^j	24.2±0.7 ^f
Leucine	69.6±0.5 ^l	58.1±0.2 ^f	65.4±0.3 ^{ij}	56.6±0.4 ^e	68.3±0.6 ^{kl}	60.4±0.5 ^{fg}
Lysine	104.2±0.9 ^o	60.0±0.1 ^g	107.5±0.9 ^p	60.2±1.0 ^g	103.3±0.1 ^{mn}	104.1±1.1 ^o
Methionine	33.6±1.3 ^j	14.2±0.0 ^{bc}	33.3±4.3 ^k	13.8±0.1 ^b	35.2±3.6 ^l	19.9±0.5 ^e
Phenylalanine	47.6±1.6 ⁿ	35.0±0.2 ^{fg}	44.5±0.3 ^l	34.7±0.2 ^f	47.4±0.9 ⁿ	44.1±0.8 ^l
Histidine	55.1±0.6 ^l	33.9±0.1 ^{cd}	52.7±0.3 ^j	32.2±0.2 ^c	54.9±0.2 ^{kl}	51.5±0.6 ⁱ

Table 3.2 (a-h). Essential amino acid composition (mg/g protein) in 44 soybean lines grown in the state of Arkansas (cont.).

(d)

Amino acid	S01-9265	UA-4805	5601T	R05-5351	S04-4729RR	TN01-235
Cysteine	30.8±4.8 ^g	13.1±0.6 ^a	14.3±0.6 ^a	16.4±0.1 ^b	19.2±1.2 ^c	15.4±0.2 ^{ab}
Threonine	21.9±0.0 ^{ef}	16.7±0.6 ^c	14.9±0.3 ^b	20.0±1.6 ^{de}	27.0±1.7 ^h	13.7±0.1 ^a
Valine	22.7±0.0 ^c	29.6±0.7 ^g	24.9±1.2 ^{de}	31.6±0.5 ^h	30.0±3.6 ^{gh}	19.7±0.9 ^b
Isoleucine	22.3±0.1 ^e	27.0±0.1 ^g	17.2±0.1 ^{bc}	29.7±0.4 ⁱ	31.4±4.2 ^j	14.6±0.0 ^a
Leucine	57.7±0.3 ^{ef}	62.3±0.5 ^{gh}	57.6±0.5 ^{ef}	53.3±0.8 ^c	65.8±3.6 ^{ij}	43.5±0.1 ^a
Lysine	61.8±0.0 ^h	96.1±0.2 ^j	102.9±0.3 ^m	50.1±0.0 ^c	56.0±3.0 ^f	48.3±0.1 ^{bc}
Methionine	16.0±0.8 ^d	34.5±3.8 ^l	33.0±3.2 ^j	13.7±0.2 ^b	28.5±0.7 ^{ij}	12.4±0.0 ^a
Phenylalanine	34.1±0.1 ^f	41.3±1.3 ^k	44.6±1.4 ^l	32.0±0.6 ^{de}	33.4±2.9 ^e	26.6±0.0 ^b
Histidine	34.5±0.0 ^d	50.0±0.4 ^{hi}	45.3±0.7 ^g	32.8±0.2 ^c	38.3±2.3 ^f	27.3±0.0 ^a

(e)

Amino acid	S04-3835RR	R05-5510	Sat (Fay)	N98 (Fdn)	Kristine	5002T
Cysteine	15.7±0.2 ^{ab}	16.6±0.0 ^b	22.0±1.9 ^{de}	14.0±0.5 ^a	16.2±0.7 ^b	14.5±0.2 ^a
Threonine	20.8±1.4 ^{de}	19.5±1.3 ^d	21.1±0.0 ^{ef}	43.5±0.6 ^m	21.7±0.7 ^{ef}	16.8±1.3 ^c
Valine	36.4±0.4 ^j	32.6±0.0 ^h	19.1±0.0 ^b	35.4±0.8 ^{ij}	30.6±1.6 ^{gh}	22.0±0.4 ^c
Isoleucine	34.6±0.2 ^l	30.9±0.0 ⁱ	16.9±0.1 ^b	34.4±0.8 ^l	27.6±1.6 ^g	19.5±1.2 ^d
Leucine	56.0±1.5 ^e	71.6±0.2 ^m	54.0±0.0 ^d	62.8±0.8 ^h	68.6±4.0 ^{kl}	67.1±0.1 ^k
Lysine	54.3±0.0 ^c	111.0±1.4 ^r	63.9±0.4 ⁱ	109.6±1.4 ^q	102.9±0.0 ^m	103.0±0.7 ^{mn}
Methionine	13.7±0.7 ^b	32.5±1.9 ^j	18.0±0.0 ^d	23.4±0.9 ^g	41.0±0.9 ⁿ	41.8±4.8 ⁿ
Phenylalanine	36.4±0.1 ^h	50.2±1.5 ^p	33.7±0.0 ^e	45.3±1.0 ^{lm}	39.0±0.1 ⁱ	38.6±0.2 ⁱ
Histidine	34.4±0.2 ^d	58.7±0.7 ^m	32.3±0.0 ^c	52.0±0.6 ^j	53.9±1.9 ^{jk}	54.5±2.1 ^k

(f)

Amino acid	IA-3017	TN-5123	R05-4481	R05-5342	R05-5494	CRR05-188
Cysteine	20.1±0.4 ^c	15.0±0.1 ^{ab}	19.7±0.1 ^c	16.7±0.0 ^b	16.6±0.2 ^b	25.5±3.3 ^f
Threonine	24.8±0.6 ^g	20.0±0.7 ^{de}	24.0±1.2 ^g	24.2±0.1 ^g	23.5±0.2 ^{fg}	19.8±0.0 ^d
Valine	35.8±0.8 ^{ij}	16.8±0.0 ^a	25.9±0.4 ^e	27.0±0.2 ^f	26.5±2.3 ^{ef}	31.7±0.0 ^h
Isoleucine	35.6±0.1 ^{lm}	16.7±0.8 ^b	22.7±0.0 ^e	28.0±0.0 ^{gh}	27.4±2.5 ^g	32.3±0.1 ^{jk}
Leucine	66.1±0.7 ^{jk}	51.9±0.8 ^b	57.9±0.0 ^{ef}	60.8±0.0 ^{fg}	57.5±0.9 ^{ef}	66.4±0.2 ^{jk}
Lysine	53.7±2.1 ^{de}	61.0±1.7 ^h	62.1±0.4 ^h	50.5±1.7 ^c	52.8±0.5 ^d	61.8±0.5 ^h
Methionine	20.2±1.0 ^{ef}	16.7±1.9 ^d	14.2±1.2 ^{bc}	20.2±2.5 ^{ef}	26.4±2.0 ^h	15.8±0.1 ^c
Phenylalanine	37.0±0.4 ^h	24.9±0.9 ^a	34.5±1.0 ^f	29.3±0.7 ^c	29.1±1.2 ^c	38.7±0.3 ⁱ
Histidine	38.2±0.5 ^f	30.0±1.1 ^b	33.1±0.2 ^{cd}	34.2±0.0 ^d	32.7±0.7 ^c	38.5±0.0 ^f

Table 3.2 (a-h). Essential amino acid composition (mg/g protein) in 44 soybean lines grown in the state of Arkansas (cont.).

(g)

Amino acid	V01-1693	V01-6338	V01-1702	S01-9364	Ozark	IA 2604
Cysteine	20.3±0.4 ^c	16.4±0.1 ^b	14.7±0.3 ^a	16.7±0.2 ^b	16.0±0.5 ^b	20.6±0.1 ^c
Threonine	22.0±0.9 ^f	17.5±1.5 ^{cd}	21.6±0.9 ^{ef}	19.8±0.0 ^d	44.2±0.7 ^{mn}	22.9±1.5 ^f
Valine	26.9±0.5 ^{ef}	23.1±1.4 ^{cd}	24.2±1.0 ^{de}	30.6±1.7 ^{gh}	32.2±0.9 ^h	31.4±0.2 ^h
Isoleucine	25.4±0.1 ^f	17.3±1.1 ^{bc}	32.3±1.7 ^{jk}	27.7±1.6 ^g	31.3±0.8 ^j	30.7±0.1 ⁱ
Leucine	61.1±0.6 ^g	53.1±0.3 ^c	58.9±0.2 ^f	65.6±0.7 ^j	64.5±1.0 ⁱ	59.5±0.1 ^f
Lysine	60.9±0.8 ^g	49.1±0.4 ^b	46.8±1.3 ^a	111.8±2.7 ^r	100.1±1.3 ^l	54.5±0.1 ^e
Methionine	21.4±0.4 ^f	20.9±2.0 ^{ef}	27.8±0.3 ^{hi}	32.6±1.5 ^j	20.3±0.6 ^{ef}	14.0±0.4 ^{bc}
Phenylalanine	33.1±1.5 ^e	26.6±0.2 ^b	26.8±0.6 ^b	41.2±2.9 ^k	48.5±0.9 ^o	33.1±0.8 ^e
Histidine	35.3±0.3 ^d	30.1±0.6 ^b	31.4±1.0 ^b	55.9±1.1 ^l	54.2±0.7 ^k	36.3±0.1 ^e

(h)

Amino acid	R05-5358	KS-5007
Cysteine	16.3±0.2 ^b	16.4±0.1 ^b
Threonine	19.7±0.6 ^d	34.2±0.2 ^j
Valine	21.8±0.9 ^c	32.1±0.1 ^h
Isoleucine	19.2±1.0 ^d	27.2±0.1 ^g
Leucine	73.3±1.1 ⁿ	63.8±0.2 ⁱ
Lysine	137.8±3.0 ^v	96.9±0.3 ^j
Methionine	39.9±0.4 ^m	26.1±0.1 ^h
Phenylalanine	36.5±1.2 ^h	44.2±0.0 ^l
Histidine	50.7±1.5 ^{hi}	54.2±0.1 ^k

Values are means ± standard deviation of triplicate analysis and those not connected by same letter in each row are significantly different from each other (P<0.05). Soybean lines: Sat (Fay) = Satellite (Fayetteville); Sat (STU) = Satellite (Stuttgart); N98 (Fdn) = N98-4445A (Foundation). (Rayaprolu *et al.*, 2015).

Table 3.3. Soybean lines with comparatively higher methionine and cysteine content (mg/g of protein) among the tested soybean lines.

Soybean line	Methionine (mg/g)	Yield Attribute
R05-4494	53.7 ± 1.4 ^a	High protein and fatty acid
R05-5491	43.5 ± 0.1 ^b	Fatty acid
5002-T	41.8 ± 4.8 ^{bc}	Check
Kristine	41.0 ± 0.8 ^{bc}	Low linolenic acid
R05-5362	35.2 ± 3.6 ^d	Fatty acid
R05-5358	39.9 ± 0.4 ^{bc}	Fatty acid
UA-4805	34.5 ± 3.8 ^d	Check
R05-4478	33.3 ± 4.3 ^d	High protein and fatty acid

Soybean line	Cysteine (mg/g)	Genetic Attribute
R05-4505	35.1 ± 0.3 ^a	High protein and fatty acid
S01-9265	30.8 ± 4.8 ^a	Low saturated fat
Satellite STU	23.7 ± 1.5 ^b	Fatty acid
CRR05-188	25.5 ± 3.3 ^{ab}	High oleic acid

Values are means ± standard deviation and those not connected with same letter are significantly different (P<0.05). STU = Stuttgart. (Rayaprolu *et al.*, 2015).

Table 3.4. Comparative analysis of essential amino acid composition (mg/g protein) of egg, milk and soy protein.

Amino acid	Egg protein*	Milk protein*	Soy protein[#]
Cysteine	24	7.9	13.1 - 35.1
Threonine	47.8	47.3	14.9 - 45.2
Valine	48.2	63.8	16.8 - 42.1
Isoleucine	51.4	68.5	14.6 - 40.2
Leucine	82.9	103.5	43.5 - 73.3
Lysine	66.4	68.3	46.8 - 137.8
Methionine	34.9	20.2	12.4 - 53.7
Phenylalanine	59.5	46.7	24.9 - 50.2
Histidine	23.5	21.8	27.3 - 58.7

*Source: FAO. Accessed at: www.fao.org/docrep/. [#]Values for soy protein are from the 44 soybean lines that are presented as a range. (Rayaprolu *et al.*, 2015).

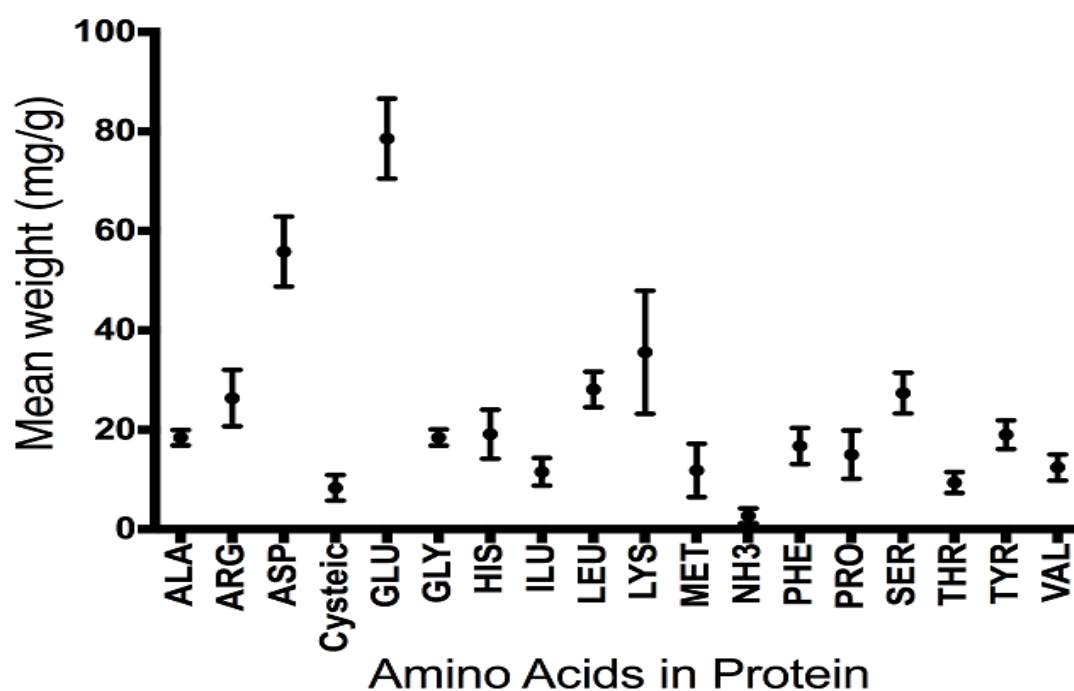


Figure 3.1. One-way analysis of amino acid profile among the 44 soybean lines.
The mean weight (mg/g of protein) from amino acid analysis are shown for each amino acid detected in the protein. (Rayaprolu *et al.*, 2015).

CHAPTER 4

Preparation of gastro-intestinal resistant peptide fractions from N98-4445A, R95-1705 and S03-543CR soybean lines, and their ACE-I inhibitory activities

(Parts of the chapter published in *Food Research International Journal*, 2013, 50: 282-288 and in the *Journal of American Oil Chemists' Society*, 2015, 92(7): 1023-1033.)

Abstract

Soy is an abundant and relatively inexpensive source of proteins which are known for their high nutritional values and excellent nutraceutical and functional properties. Enzymatic digestions of soy allergenic proteins are of interest, since, enzymatic digestion is done not only to get rid of a large portion of allergenic proteins but also obtain peptides. Potentially hypoallergenic peptides thus obtained may be considered for unexplored bio-functional attributes. In this study protein isolates were prepared from three selected soybean lines, R95-1705 (high in protein), N98-4445A (high in oleic acid and protein) and S03-543CR (high in oleic acid and protein) with highest purity (90%). Conditions were optimized for enzymatic digestion of the protein isolate with Alcalase (subtilisin) using response surface methodology. The protein hydrolysates obtained were exposed to simulated gastric and intestinal juices for testing their resistance to human digestive enzymes (GI). The resistant hydrolysates were then separated based on molecular cut-off sizes into three different fractions using ultrafiltration membranes. This resulted in GI resistant protein fractions with distinct molecular cut-offs: <5kDa, 5-10kDa and 10-50kDa that were freeze dried. Furthermore, the GI resistant fractions were tested for angiotensin-I-converting enzyme (ACE-I) inhibition activity. The 5-10kDa protein hydrolysate (500 µg/mL) from R95-1705 showed highest inhibition of the ACE-I by 49 %. The results from this study will promote the use of high oleic acid soybeans as a source of protein and peptides with functional activities.

Introduction

Proteins from plant sources are part of human diet which are incorporated into several products in the food industry. This is due to their functional properties which are beneficial for maintaining human health. Soybean is a widely cultivated legume for edible oil and the co-product or residue after oil extraction is chiefly used as meal in farm animal feed. The soybean meal or oil extracted residue is mainly known for the protein content which has been increasingly consumed by humans as a good substitute for animal protein. There are various methods to separate native proteins from seed flour without denaturing it and retaining its properties. The seed flour can be treated with enzymes to prepare the protein isolate by digesting the starch with alpha-amylase (Shih and Daigle, 1997, Tang *et al.*, 2002). However, alkaline hydrolysis is the simplest method of solubilizing the protein, and separating it from the starch. Researchers have shown that soy proteins lose their allergenic properties when digested with proteases. The resulting hydrolysates when tested for their binding abilities to either a monoclonal antibody or sera obtained a negative result (Obata *et al.*, 1998; Yamanishi *et al.*, 1996)). Obata *et al* (1998) reported reduction of allergenicity in tofu (soybean surd) after enzymatic digestion. Reduction in allergenicity after enzymatic digestion of soybean grains was demonstrated by Yaminishi *et al* (1996). This also leads to the generation of pure peptides (Kannan *et al.*, 2010).

Alcalase and liquipanol are two proteases with broad specificity that can cleave the proteins either at Arg-Arg residues or at uncharged carboxy terminal end residues. Enzyme specificities provide the ability to cleave the substrate proteins at specific sites. This information for various proteases commonly used in food protein hydrolysis are given in Table 4.1. Bioactive peptides thus released can potentiate biological effects with even a fraction of them being

absorbed in our body. However, specificity and extent of hydrolysis are important factors that need to be controlled to improve functional properties of food proteins and peptides. The solubility of proteins increases with increase in degree of hydrolysis (DH) possibly due to the reduction in molecular weight and an increase in the number of polar groups (Nielsen, 1997). Degree of hydrolysis represents the proportion of peptide bonds hydrolyzed and it is calculated using the equation $DH = (h_n \times 100)/h_{tot}$ where h_n is number of peptide bonds hydrolysed and h_{tot} is total number of peptide bonds present in the parent protein. The units of h and h_{tot} are both meq/gm (Adler-Nissen, 1986). The h_{tot} of a protein is calculated from the amino acid composition of the specific protein being hydrolyzed (Adler-Nissen, 1986). During hydrolysis, a new carboxyl and a new amino group is released for each cleaved amide bond. The number of hydrolyzed peptide bonds can be calculated by determining the number of newly formed N-terminal or C-terminal groups in hydrolysates. The amount of released α -amino groups can be measured by using reagents that react specifically with amino groups, yielding compounds that can be detected spectrophotometrically. Reagents generally used are o-phthalaldehyde (OPA), ninhydrin, and trinitrobenzenesulphonic acid (TNBS).

Proteins have proved to be excellent sources for bioactive peptides, especially in reducing hypertension, which is a precursor for heart disease (Leppälä, 2000, Palatini and Julius, 2009). Inhibition of angiotensin-1-converting enzyme (ACE-I) activity has a potential link to a hypertension lowering effect, by preventing the conversion of angiotensin I to Angiotensin II, where the latter compound is responsible for contracting the epithelial layer of arteries causing an increase in blood pressure (Boudier *et al.*, 1992). Previous studies have shown that peptides derived from soybean protein with limited enzymatic hydrolysis possess ACE-I inhibitory activity (Wu and Ding, 2002, Kuba *et al.*, 2005) and other bio-activities (Rayaprolu *et al.*, 2013).

There have been no studies determining the presence of higher than normal amounts of methionine from high oleic acid soybean meals, or the influence of amino acid content and sequence which can elicit significant biological activities. The objective of this study was to test for ACE-I inhibitory activity of the peptide fractions obtained from previous research. Food-derived proteins and peptides have already been considered for interesting applications as medical foods or pharmaceutical preparations. This is the first time that amino acid analysis of soybean lines with varying oleic acid composition and ACE-I inhibitory activity assessment of Alcalase enzyme-derived peptides from their protein isolates has been studied.

Materials and Methods

The seeds of 44 soybean lines, N98-4445A, R95-1705 and S03-543CR, were obtained from Dr. Pengyin Chen, Plant breeder and Professor, Department of Crop Soil and Environmental Sciences, University of Arkansas. Food grade enzyme Alcalase 2.5L (EC 3.4.21.62) was purchased from Novozyme (Bagsvaerd, Denmark) for preparing the protein hydrolysates.

Preparation of soy meal and protein extraction

Seeds of all three soybean lines were provided by Dr. Pengyin Chen (Soybean breeder, University of Arkansas, Fayetteville, AR, USA). The seeds from two high oleic acid soybean lines (N98-4445A and S03-543CR) and one high protein line (R95-1705) were ground and sieved (250 microns) to obtain uniform particle size. The lipid was extracted using the solvent *N*-hexane (1:3 w/v ratio of flour: solvent) and the lipid content of the meal was determined by the Soxhlet method (AOAC, 1995). The meals were suspended in de-ionized (DI) water (10% w/v) and protein isolates were prepared by alkali extraction method (pH 9.5 at ambient temperature

for 3 hr) using 3N NaOH solution. The supernatants were separated by centrifugation at 3000 g for 15 min and the residue was re-extracted by repeating the process. The collected supernatants for each soy line were mixed to prepare the isolate using isoelectric precipitation at pH 4.5. The final pH was adjusted to 7.0 and the three SPIs were freeze dried. The protein percent was determined by the Kjeldahl method based on a conversion factor of 6.25 for Nitrogen (AOAC, 1995).

Optimization of conditions for enzymatic hydrolysis with Alcalase

The conditions for optimization of enzymatic hydrolysis using Alcalase were determined based on the Box-Behnken Design of the Response Surface Methodology (RSM) (Ma and Ooraikul, 1986). The SPI was mixed with DI water (1:10 w/v) and homogenized. Enzymes were purchased from Novozyme Inc. (Bagsvaerd, Denmark). The suspensions were subjected to treatments involving four factors at three levels in the design viz., pH (6.5, 7.5 and 8.5), time (30, 60 and 90 min), temperature (55, 62.5 and 70 °C), and enzyme concentration (1, 3.5, and 5 Anson units). The enzyme was inactivated at 85 °C for 3 min; the hydrolysates were cooled to ambient temperature and centrifuged at 3,000 g for 10 min. The degree of hydrolysis (DH) was determined using the OPA method in the supernatants that were collected. DH is defined as the percentage of peptide bonds cleaved from the total number of peptide bonds in the protein (Mahmoud *et al.*, 2006). Ten micro liters of the samples were added to 3 mL of OPA reagent and the solutions were left to stand for 2 min before taking the spectrophotometer reading at 340 nm (Adler-Nissen, 1978). Blank was prepared using 400 µL of DI water in 3 mL of OPA reagent. 400 µL of 0.01% serine solution in 3 mL of the reagent was used as a standard. The optical density (OD) values of all the samples were recorded and the following formulae were used to calculate the degree of hydrolysis (Neilsen *et al.*, 2001):

$$\text{Serine-NH}_2 = (\text{Sample}_{\text{OD}} - \text{Blank}_{\text{OD}}) / (\text{Standard}_{\text{OD}} - \text{Blank}_{\text{OD}}) * 0.9516 * V * 100 / X * P$$

Where, V = sample volume; X = amount of protein in the sample; and P = protein % in sample.

Hydrolyzed peptide bonds $H = (\text{Serine-NH}_2 \cdot \beta) / \alpha$, where α and β value for soy are 0.248, and 0.97 respectively. Degree of hydrolysis $DH = H / H_{\text{tot}} * 100$, where H_{tot} is the total number of peptide bonds in soy, which is 7.8 (Neilsen *et al.*, 2001).

Preparation of gastro-intestinal environment resistant peptide hydrolysates with molecular sizes, <5 kDa, 5-10 kDa, and 10-50 kDa

The simulated gastro-intestinal (GI) juice was prepared mimicking the human GI tract secretions for treating the protein hydrolysates after Alcalase treatment (Kannan *et al.*, 2008). Resistance to gastric juice was determined by treating the protein hydrolysates with Pepsin (0.32 g/100 mL) after adjusting the pH to 2.0 using 3N hydrochloric acid solution and stirring for 1hr at 37 °C. The enzyme was inactivated by adjusting the pH to 7.2 and the contents were centrifuged at 3000g for 10min to separate the supernatant. The pH of supernatant was adjusted to 8.0 using 3N sodium hydroxide solution and enzyme Pancreatin (0.1 g/100 mL) was added to test for resistance against the intestinal juice. The protein hydrolysates were stirred for 1hr at 37 °C before inactivating the enzyme in water bath at 85 °C for 3min. The GI treated peptide hydrolysates were centrifuged at 3000 g for 10 min and the supernatants were passed through the membrane filters of Romicon ultrafiltration system (Koch membrane systems, Wilmington, MA, USA) with molecular cut-off sizes of 5, 10 and 50kDa. The hydrolysates were passed through each column starting with 5 kDa as the permeates were collected and retentates were passed through 10 kDa and then through a 50-kDa column following the similar process. The specific peptide fractions, <5, 5–10 and 10–50 kDa, were obtained as a results of ultrafiltration process. All the fractions were freeze dried and stored at 5 °C. Further study included testing the peptide

fractions for ACE-I inhibitory activity which was compared with that of non-GI resistant fractions.

ACE-I Inhibitory Activity Assay

A modified method of Cushman and Cheung (1971) was used to conduct the ACE-I inhibition activity assay. Protein hydrolysates at 500 µg/mL (30 µL), N-hippuryl-L-histidyl-L-leucine (HHL: 150 µL, 6.5mM), and ACE-I (25 µL, 2.5 mU) were incubated at 37 °C for 1 h. Hydrochloric acid (250 µL, 1N) and ethyl Acetate (1.5 mL) were added to stop the reaction, and the contents were mixed by vortexing before centrifugation at 2000 g for 5 min. One milliliter of the top layer (containing hippuric acid extracted with ethyl Acetate) was collected, and ethyl Acetate was removed using a roto vapor. The residual hippuric acid is dissolved with deionized water (1 mL) and absorbance was measured at 228 nm. Captopril (10mM) was used as positive control while a solution containing ACE-I and HHL was used as blank solution. Inhibition of ACE-I was calculated using the formula:

$$\% \text{ inhibition} = 1 - [(Ab_{\text{Test}} - Ab_{\text{Sblank}}) / Ab_{\text{Sblank}}] \times 100.$$

The % ACE-I inhibition was defined as the percentage of ACE-I activity inhibited by a specific amount of peptides. A dose response study was conducted for the fraction with highest ACE-I inhibition using fraction concentrations between 200 and 1200 µg/ mL with increments 200 µg/ mL using the above method. The data from the dose response was used to determine the IC₅₀ value for the peptide with highest activity.

Purification of the fractions with highest ACE-I activity

A reverse phase HPLC analysis of the peptide fractions with highest ACE-I activity was conducted with a two solvent system. Solvent A was prepared with 0.1% Trifluoro Acetic acid in Millipore water and solvent B was 50% Acetonitrile in water with 0.1% Trifluoro Acetic acid. A

linear solvent gradient was used for a 75 minute run with a 1 mL/min flow rate. Solvent A had a gradual decrease in gradient from 100% to 0% while solvent B had 0 to 100% gradient over the 75 min run. A C18 protein separation column (Sigma Aldrich, MO, USA) was used in isolating the single peptides.

Statistical analysis:

The JMP software from SAS institute (Cary, NC) was used for the experimental design and all the statistical analyses. Statistical analysis of the effect of four factors viz., pH, enzyme concentration, time and temperature on the degree of hydrolysis was conducted using the Box-Behnken design of the response surface method. The optimized conditions for the degree of hydrolysis were determined based on the 3 point prediction profiler and a P value <0.05 was used for all the analyses.

Results and Discussion

Purity of protein isolates

Previous literature has shown that alkali extraction is an optimized method to isolate proteins for studying their functional properties (Okezie and Bello, 1988). Hence, protein from the three soy meals was extracted at a lower pH of 9.5 at ambient temperature and the process was repeated to maximize the yield without affecting the protein quality. Protein contents of the soybean lines were ~91 % for S03-543CR and N98-4445A, and ~93 % for R95-1705 (Table 4.2) signifying high protein purity.

Optimization of conditions for enzymatic hydrolysis

The objective was to hydrolyze the proteins present in the prepared high-purity soy meal protein isolates to liberate peptides that may be of bioactive value. In order to accomplish

hydrolysis of the proteins, a Box-Behnken RSM statistical design was chosen to evaluate combinations of variables such as pH, temperature, enzyme concentration and time of hydrolysis at three levels for optimal hydrolysis (Table 4.3). Degree of hydrolysis (DH) was determined for each combination and an optimum value was selected to achieve optimal hydrolysis of the proteins that resulted in both high and low molecular weight fractions. The amount of enzyme added and the pH of the suspension had shown significant effect on the DH which ranged between 18.8 % and 57.4 %. A DH of 30 % was selected as the optimum condition at pH 7, enzyme concentration of 1.675 AU, temperature of 55 °C for 45 min (Figure 4.1). The optimized conditions were selected to allow formation of considerable size peptides and to prevent total digestion of the protein when exposed to the gastro-intestinal juices.

GI resistance and preparation of peptide fractions

While larger size peptide fractions are known for their functionality in food products (Wu *et al.*, 1998), smaller size peptide fractions were also considered as they have been shown to have higher potency (Wu and Ding, 2002) for wider applications. Simulated GI resistance demonstrated the resistant of peptides to digestion in the GI tract and could be available for absorption through the intestine. Three molecular cut off membrane columns of 5, 10 and 50kDa were used to fractionate the peptide hydrolysates and permeates and retentates were collected from each column. The nine specific fractions of <5kDa, 5-10kDa and 10-50kDa for each soy line were collected to test the cancer cell inhibitory property.

Activity of Protein Hydrolysates.

Three soybean lines, S03-543CR and N98-4445A (with high oleic acid) and R95-1705 (high yield and non-GMO), were selected based on their high protein content. The alkali extraction method provided >90 % protein yield in the isolates (yield: >83 % d. b. by mass

balance) which were used to prepare the protein fragments. The optimal conditions to accomplish the Alcalase enzymatic hydrolysis of the proteins in order to derive peptides of varying sizes were achieved using a statistical design for a 30 % degree of hydrolysis to derive protein hydrolysates or peptides of varying sizes (Rayaprolu *et al.*, 2013). The ultrafiltration of GI-resistant protein hydrolysates using molecular cut-offs columns—5, 10 and 50kDa provided the fractions <5, 5-10, 10-50 and >50kDa (yield: 1.8–2.1 % d. b. by mass balance, derived from the isolate) for each soybean line with protein content ranging between 89 and 92 %. Initial screening showed comparatively poor ACE-I inhibition by the >50kDa fractions which were eliminated during further testing. The non-GI fractions showed overall low ACE-I inhibition when compared to the GI fractions which was in agreement with previous research (Kannan, 2009).

A concentration of 500 µg/mL was used for the study where the results showed overall low activity in comparison to the positive control, captopril (approximately 75 % inhibition). However, the highest inhibition among the fractions was 48.9% by the 5-10kDa obtained from R95-1705 soybean line (Figure 4.2), and this fraction was chosen for the dose response study to determine the minimum inhibitory concentration. The <5-kDa fraction from the high oleic acid soybean line N98-4445A showed an inhibition of 42.2 ± 1.3 % which was the only other significant activity observed against the ACE-I enzyme among all the protein hydrolysate fractions. Peptides of both large and small sizes have shown bioactivities in previous studies and have exhibited functionalities that can be used in food products (Wu *et al.*, 1998, Wu and Ding, 2002). The hydrolysates tested for GI resistance could potentially be available for absorption through the intestine when consumed as food and elicit the health beneficial bioactivities in the target tissues. Researchers have shown that peptides of various molecular sizes are absorbed

through the intestinal wall but the ability of absorption decreases with an increase in molecular size (Roberts *et al.*, 1998).

Dose response study of the fraction with highest activity and purification using chromatography

A dose response study of the 5-10kDa fraction (R95-1705) revealed an increase in ACE-I inhibitory activity as the dosage of the fractions increased (Figure 4.3). The highest inhibition was achieved at 1200 µg/mL concentration (75.5 ± 2.8 %) which was not significantly different from that shown by 1000 µg/mL concentration (72.4 ± 1.4 %). An increase in ACE-I inhibition was observed at ≥ 800 µg/mL concentration, although the highest inhibition by the fractions was significantly lower in comparison to the positive control. The dose response provided the inhibitory concentration at 50 % activity (IC₅₀) of the 5-10kDa protein fraction from R95-1705 to be 563 µg/mL. These results are significant as R95-1705 is a non-GM soybean line, which can have a potential impact on its utilization in foods or supplemental therapeutics. The ACE-I inhibitory activity can be attributed to the pool of peptides from 5-10kDa and their amino acid sequences. However, other studies have reported significant ACE-I inhibitory activity by similar molecular size peptide fractions which were derived from marine protein sources (Lee *et al.*, 2010). Previous studies from the current research group have shown the ability of large molecular size peptide fractions (>50 kDa) to have ACE-I inhibition (Lassissi *et al.*, 2014). Researchers have also reported ACE-I and atherosclerosis inhibition by hydrolysates obtained from both glycinin and β -conglycinin fractions of soy protein (Kuba *et al.*, 2005, Adams *et al.*, 2004). Hence, the peptide fraction from R95-1705 (5-10kDa) with anti- ACE-I activity may have been derived from either the glycinin (11S) or β -conglycinin (7S) fractions of the proteins which needs further examination.

Several factors can be attributed to the substantial anti-ACE-I activity that can be studied, starting with the purification of the peptide pool from the 5-10kDa fraction from R95-1705 soybean line. The HPLC analysis of the peptide fraction showed 5 individual peptides which were separated by the elution time on the column (Figure 4.4). The solvent system and the gradient used were efficient in separating the 5 peptides in the fraction.

Studies have also shown that protein hydrolysates and peptides derived from various food sources possess anti-ACE-I activities (Lee *et al.*, 2010, Balti *et al.*, 2010, Lassissi *et al.*, 2014). Although most researchers tested (pure) peptides derived from either fermentation or enzymatic hydrolysis, very few studies have demonstrated the bioactivity of GI-resistant protein hydrolysates which contain a pool of peptides. Preparation of hydrolysates is economical in comparison to derivation of pure peptides. Peptide fractions can provide synergistic effects and have shown multiple bioactivities (Kannan *et al.*, 2008, Rayaprolu *et al.*, 2013).

Conclusion

The protein fraction from the R95-1705, a non-GM soybean, showed highest ACE-I inhibition. This is the first time ACE-I inhibition has been achieved with GI-resistant non-GMO soy protein hydrolysates derived by enzymatic digestion of high purity protein isolates. The 5-10kDa protein fraction at a higher dose presented an enhanced ACE-I inhibitory activity. In conclusion, this study provides amino acid composition of soybean lines that are grown for definite yield attributes and demonstrates functional activity of protein/peptide fractions derived from selected soybean lines.

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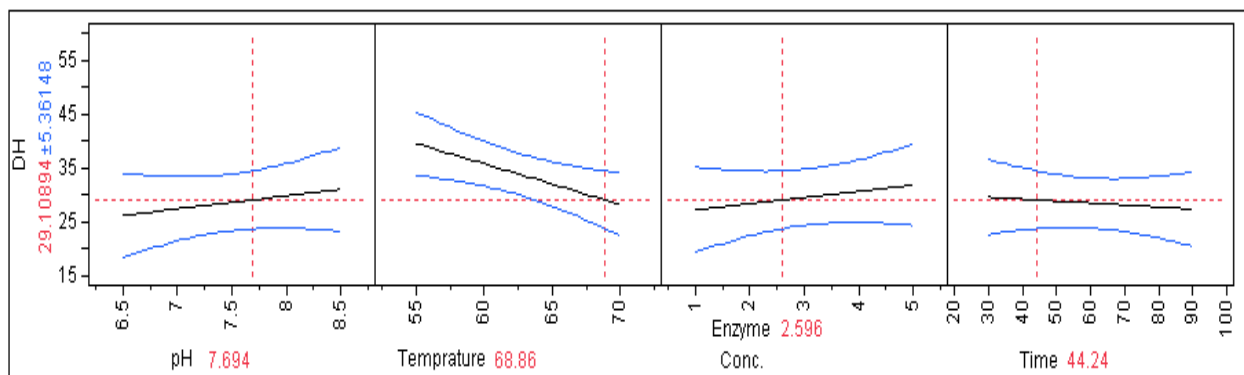


Figure 4.1. The prediction profiler of the JMP showing the optimized conditions. (DH=degree of hydrolysis, Enz=Enzyme concentration). (Rayaprolu *et al.*, 2015).

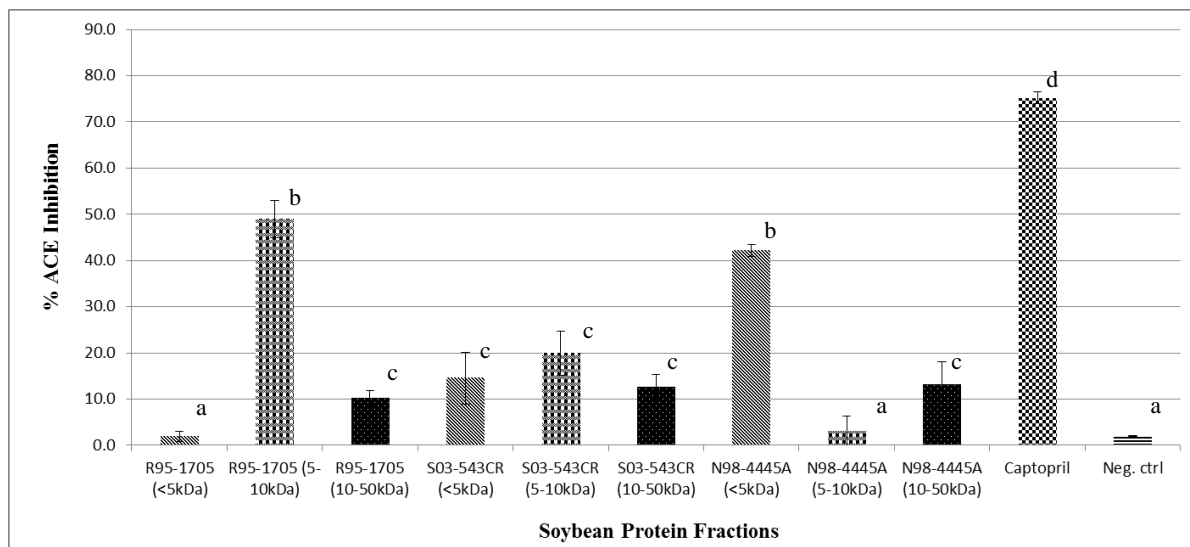


Figure 4.2. The ACE-I inhibitory activity of soybean peptide fractions at 500µg/mL concentration. Values are means \pm standard deviation. Bars not connected with same letter are significantly different ($P < 0.05$). (Rayaprolu *et al.*, 2015).

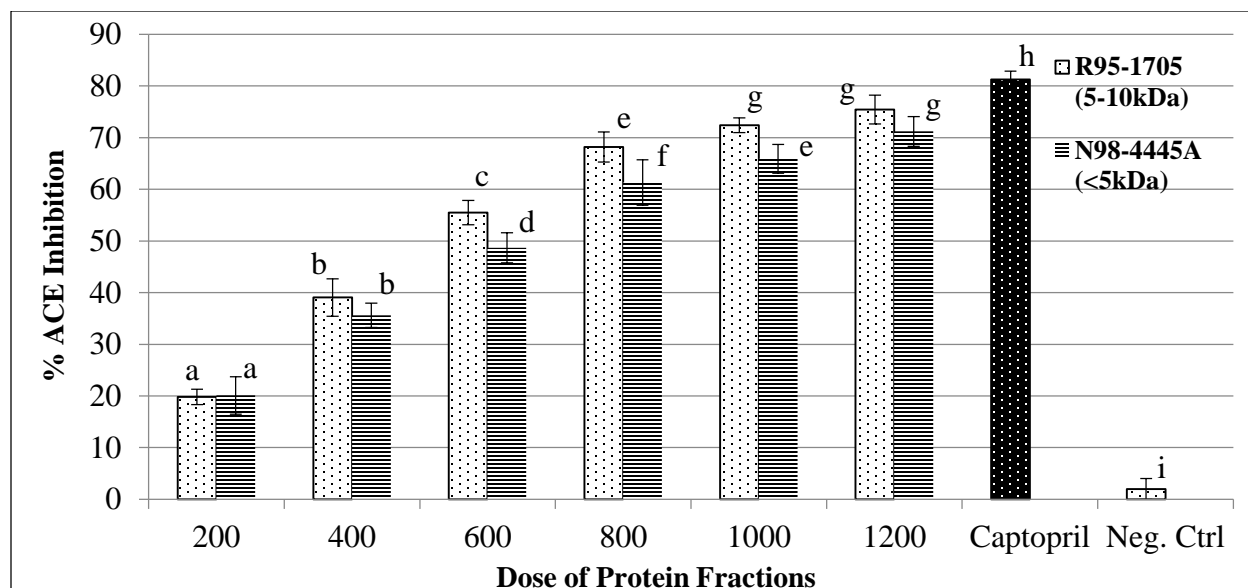


Figure 4.3. Dose response study of 5-10kDa and <5kDa peptide fractions from R95-1705 and N98-4445A soybean lines on ACE-I inhibitory activity. Captopril 10mM – Positive control. Negative Ctrl – 1.0% saline. Bars are means \pm standard deviation and those not connected with same letter are significantly different ($P<0.05$). (Rayaprolu *et al.*, 2015).

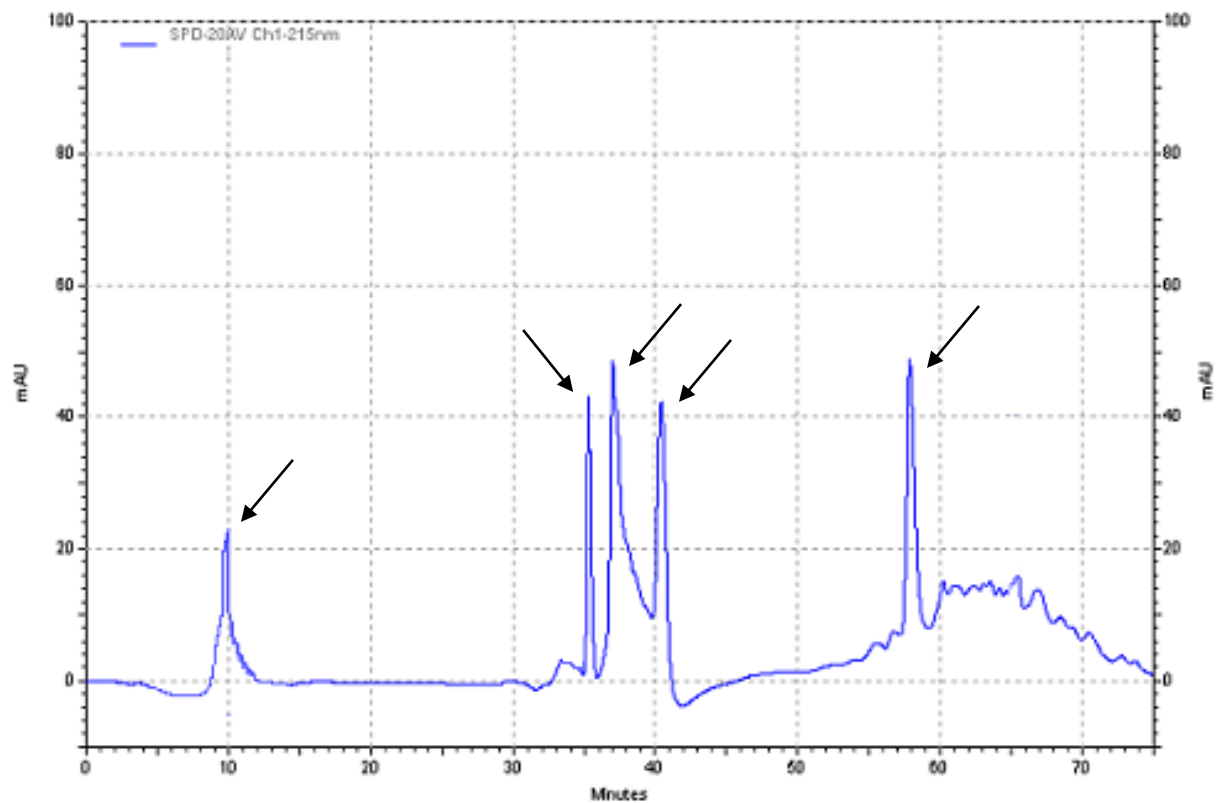


Figure 4.4. Purification of the 5-10kDa peptide fraction from R95-1705 using HPLC method. The arrow heads represent 5 individual peptides present in the 5-10kDa fraction which showed combined activity against ACE-I. (Rayaprolu *et al.*, 2015).

Table 4.1. Types of proteases and specificity of these enzymes used for protein hydrolysis in general (Adler-Nissen 1993).

Enzyme	Type of protease	Specificity
Liquipanol	Cysteine	Broad
Bromelain	Cysteine	Broad, prefers Arg-Arg peptides
Alcalase	Serine	Broad, prefers uncharged residue's carboxyl site
Protex 6L	Serine	Broad for peptide bonds
Trypsin and Chymotrypsin	Serine	Lys, Arg, Phe, Trp, Tyr, Ala
Carboxypeptidase	Metallo	Terminal amino acids at C-terminus with exceptions: Pro, Arg, Lys

Table 4.2. Protein content (purity) in the isolate extracted from three soybean lines (N98-4445A, R95-1705 and S03-543CR).

Soybean line	Protein % (dry basis)
S03-543CR	90.82 \pm 0.61 ^a
N98-4445A	91.6 \pm 0.95 ^a
R95-1705	93.34 \pm 1.29 ^b

Values (means \pm standard deviation) not connected with same letters are significantly different (P<0.05). (Rayaprolu *et al.*, 2015).

Table 4.3. The Box-Behnken design of the surface response method to determine the optimal degree of enzymatic hydrolysis.

S.No	Pattern	Enzyme	pH	Time (min)	Temperature (°C)
1	-----	1	6.5	30	55
2	-----+	1	6.5	30	70
3	----+-	1	6.5	90	55
4	----++	1	6.5	90	70
5	a000	1	7.5	60	62.5
6	-+---	1	8.5	30	55
7	-++-	1	8.5	30	70
8	-++-	1	8.5	90	55
9	-+++	1	8.5	90	70
10	0a00	3.5	6.5	60	62.5
11	00a0	3.5	7.5	30	62.5
12	000a	3.5	7.5	60	55
13	0	3.5	7.5	60	62.5
14	0	3.5	7.5	60	62.5
15	000A	3.5	7.5	60	70
16	00A0	3.5	7.5	90	62.5
17	0A00	3.5	8.5	60	62.5
18	+---	5	6.5	30	55
19	+---+	5	6.5	30	70
20	+--+	5	6.5	90	55
21	+---+	5	6.5	90	70
22	A000	5	7.5	60	62.5
23	++--	5	8.5	30	55
24	++-+	5	8.5	30	70
25	+++--	5	8.5	90	55
26	++++	5	8.5	90	70

The pH, time, temperature and enzyme concentration (Enz) are the independent variables, each with three levels. The column S. No. shows the number of experimental runs while 'Pattern' column shows the design for each experiment where '-' stands for the lowest level, '0' is for the middle and '+' is for the highest level for each variable.

CHAPTER 5

Peptides Derived from High Oleic Acid Soybean Meals Inhibit Colon, Liver and Lung Cancer Cell Growth

(Part of the chapter published in *Food Research International Journal*, 50: 282-288.)

Abstract

Soybean meal, a co-product after oil extraction from seeds, is rich in protein. Our objective was to utilize this co-product, obtain gastrointestinal (GI) resistant peptides from the isolated protein, and test for bioactivity against colon, liver and lung cancer cell lines. N98-4445A, S03-543CR high oleic acid soybean lines, and R95-1705 high protein soybean line were used for this study. Protein isolates were prepared at alkaline pH and hydrolyzed using Alcalase enzyme to generate peptide hydrolysates. After determining gastrointestinal resistance of the peptide hydrolysates they were fractionated into definite molecular sizes of <5 kDa, 5-10 kDa, and 10-50 kDa and tested against human colon (HCT-116, Caco-2), liver (HepG-2) and lung (NCL-H1299) cancer cell lines. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cytotoxicity assay was performed to test *in vitro* cancer cell viability upon treatment with peptide fractions. The peptide fractions from N98-4445A and S03-543CR lines showed cell growth inhibition of 73 % of colon cancer (HCT-116), 70 % of liver cancer cells and 68 % of lung cancer cells. Dose response showed that the peptides had significant inhibitory effect at higher concentrations (1000 µg/mL to 600 µg/mL) and gradually decreased with decreased dosage (500 µg/mL to 100 µg/mL). Soybean peptide fractions can thus be a source of bioactivity against colon, liver and lung cancer cell proliferation.

Introduction

Soybeans are grown for commercial edible oil, and among the genetic lines the high oleic acid (HOA) lines are in demand from the industry due to high oleic to linolenic acid ratio that is more stable in food products (USDA 2009). The HOA soy lines have genetically manipulated fatty acid content in the seeds with higher amounts of mono-unsaturated oleic acid as a substitute for poly-unsaturated linoleic acid (Cahoon, 2003 and Hyten *et al.*, 2004). High oleic acid oil causes low rancidity in the foods prepared with the soybean oil, giving them a longer shelf life. Studies have also shown an increase in essential amino acid, lysine in the hybridized high oleic acid soybean seeds compared to the parents (Kinney, 2007). This is an essential criterion since the residue after oil extraction from the seeds, soy meal, is a rich source of protein with the essential amino acids except for the sulfur containing amino acid methionine. Soybean protein is an excellent healthy substitute for animal protein and is used as a protein supplement in muscle building. While processed animal meats are known to cause health concerns including cancer and heart disease due to the presence of nitrosamines, soy protein has been proved to promote health without causing any detrimental health conditions (Norat *et al.*, 2001).

Cancer is the second leading cause of death in the United States after heart disease. The Centers for Disease Control and Prevention has claimed an estimated 577,190 cancer deaths in 2012 (ACS, 2012). Approximately 1.6 million new cancer cases were to be diagnosed of which approximately 9 % are colorectal and approximately 29,000 new cases of liver and related organs are expected in 2012. According to American Cancer Society an estimated 14 % of cancer diagnoses in 2012 are related to lung and bronchus while, colorectal cancer is ranked third among all cancer deaths in the United States (ACS, 2012). Treatment of cancer is highly expensive involving drugs that have adverse side effects and toxicity complications. Cheaper

alternative methods using bioactive peptides can have better prospects in the economics of cancer prevention and therapy. These bioactive peptides are derived from food ingredients and are acceptable due to their non-toxic nature (Silva-sanchez *et al.*, 2008). Bioactive peptides from food sources like egg protein and milk protein with health promoting properties have been studied extensively in the recent years (De Mejía and De Lumen, 2006). Peptides from fermented soy products including Tempeh and Natto (Gibbs *et al.*, 2003) and those formed after enzymatic digestion of protein from soybeans and rice bran have shown bio-activity against cancer (Zang *et al.*, 1998; Kannan *et al.*, 2008). Researcher also studied peptides derived from bacterial fermentation of milk to determine their bioactivity (Algaron *et al.*, 2004). However, no literature information on bioactivity is available on protein or peptides derived by enzymatic hydrolysis from high oleic acid soybean meals.

The primary objective of this research was to derive peptides from the soybean meal of high oleic acid soybean lines N98-4445A and S03-543CR and compare the cell proliferation inhibition with that of the peptides prepared from a high protein soybean line R95-1705. Alcalase enzyme was used to hydrolyze the isolated protein, since it is extensively studied and used in the industry (Gupta *et al.*, 2002). In this study, peptide hydrolysates were prepared from high oleic acid soy protein isolates (SPIs), tested for gastrointestinal resistance, fractionated based on molecular size, and tested for multiple site cancer inhibiting activities.

Materials and methods

Preparation of Protein Isolate and Enzymatic Hydrolysis to Derive Gastro-Intestinal Resistant Peptide Fractions

Seeds from the three selected (based on high protein content) soybean lines (high oleic acid: N98-4445A, S03- 543CR; high yield: R95-1705) were ground, defatted and passed through a 60-mesh (250 µm particle size) sieve. Suspensions of the flours were prepared to extract the protein at an alkaline pH of 9.5 precipitated at isoelectric pH of 4.5 to obtain the protein isolates. The protein isolates were digested using the enzyme Alcalase 2.4L under optimal conditions to derive varying size protein hydrolysates (Rayaprolu *et al.*, 2013). The hydrolysates were then passed through a simulated gastro-intestinal (GI) environment using the enzymes, pepsin and pancreatin, at 37 °C to derive GI-resistant protein hydrolysates. Ultrafiltration molecular cut-off membrane columns were used to collect the peptide fractions, <5, 5–10 and 10–50kDa, which were freeze-dried and stored at 4 °C.

Cell proliferation inhibition assay against human cancer cell lines and dose and time response study

The GI resistant peptide hydrolysates were tested on human cancer cell lines and normal human colon and lung cell lines for toxicity. The Caco-2 (ATCC number: HTB-37), HCT-116 (ATCC number: CCL-247) colon epithelial cancer cells and CCD-18Co normal colon fibroblast cells (ATCC number: CRL 1459); HepG-2 (ATCC number: HB-8065) liver epithelial cancer cells; NCL-H1299 (ATCC number: CRL-5803) lung cancer cells and LL 47 (MaDo) normal lung fibroblasts (ATCC number: CCL-135); and the culture media were purchased from ATCC (Manassas, VA, USA). The cell lines were cultured in 25 cm² flasks by adding 10mL of media and incubating at 37 °C with 5% CO₂ for 24 - 48 hours. Stock cultures were prepared (approximately between 200,000 to 2,000,000 cells) by trypsinizing the monolayer of cells with 3 mL of Trypsin-EDTA reagent (0.1% Trypsin + 0.53mM EDTA solution).

The prepared stocks were used to culture the cells in 25 cm² flasks, trypsinized and transferred to 96-well tissue culture plates with fresh media and incubated at 37 °C with 5% CO₂ for 48 hr. The peptide fractions in solution (800 µg/mL) were added to the wells along with fresh media and the plates were incubated at 37 °C with 5% CO₂ for 24-36 hours. For the dose response study, 10-50 kDa peptide fractions from N98-4445A soy line were prepared with varying concentrations ranging from 100 µg/mL to 1000 µg/mL and compared with 200 µg/mL Genistein. The plates were observed for disruption of the mono layer over a period of 18 to 72 hours. The 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) (Promega Inc., Madison, WI, USA) assay was conducted to determine the cell growth inhibition by the peptides. Wells untreated were considered as the negative control and the test was done in triplicate for all cell lines. The mitochondria of the live cells metabolize the MTS reagent and form a soluble substance called Formazan. The amount of formazan liberated in solution formed by the cancer cells was used to determine the number of live cells by measuring the absorbance at 490 nm in a micro plate reader (Bio-Rad Inc., Hercules, CA, USA). The % inhibition of the cancer cell proliferation by the GI resistant peptide fractions were calculated as:

$$\% \text{ Inhibition} = 1 - [(Abs_{Test} - Abs_{NegCtl}) / (Abs_{PosCtl} - Abs_{NegCtl})] * 100$$

Where, Abs_{Test} = absorbance of the test sample, Abs_{NegCtl} = absorbance of the negative control, and Abs_{PosCtl} = absorbance of positive control (Genistein).

The hydrolysate with highest inhibitory activity was also tested for cell toxicity using the MTS assay against normal human colon cell line. The dose response test was conducted for the fraction with highest activity against cancer cell proliferation at a dosage ranging from 100 to

1000 µg/mL. This was used to calculate the minimum effective concentration of the peptide fractions against the cancer cells.

Statistical analysis

The Student's t test with JMP software from SAS institute (Cary, NC) was used for the statistical analyses: analysis of variance, means and standard deviations (P value <0.05).

Results and discussion

Preparation of GI resistant peptide fractions under optimal conditions

Protein extraction under alkaline conditions yielded highest purity (91-93%) as per previous research (Okezie and Bello, 1988). The Box-Behnken RSM statistical design was effective in evaluating the optimal conditions for enzymatic hydrolysis of protein isolates from the three soybean lines based on degree of hydrolysis. A DH of 30 % was selected as the optimum condition at pH 7, enzyme concentration of 1.675 AU, temperature of 55 °C for 45 min to derive high and low molecular weight protein fractions. This allowed for formation of considerable size peptides that can resist complete digestion when exposed to the gastrointestinal juices.

Peptides of varying sizes were shown to provide functionality in food products (Wu *et al.*, 1998, Wu and Ding 2002). The resistance to GI juices by the peptides demonstrated the potential for getting absorbed through the intestinal lining and to elicit tissue level functional properties. Ultrafiltration technology proved adequate for separating the GI resistant peptides based on their molecular sizes. The three membrane size cut-offs, <5 kDa, 5-10 kDa and 10-50 kDa, from each soy line were collected to test the cancer cell inhibitory property.

Inhibition of cancer cell proliferation

Colon cancer cell growth inhibition (Caco-2 and HCT-116)

Two colon cancer cell types were chosen to test the effectiveness of the peptide fractions derived from the three soy meals. While Caco-2 represents cells derived from cancerous colon, HCT-116 represents a colon with tumorigenic pathology. The Caco-2 cells showed slower growth in vitro (24 to 48 hr) when compared to HCT-116, HepG-2 and NCL-H1299 cell lines (18 to 24hr) which was based on the rate of mono layer formation. The high molecular sized fractions (10-50 kDa) from S03-543CR line showed 50% inhibition on Caco-2 cancer cells (Figure 5.1) which was not significantly different from that of the low molecular weight <5 kDa fractions from N98-4445A, while the positive control inhibited 63% of the cancer cells . The highest inhibitory activity on Caco-2 cells was documented by the <5 kDa peptide fractions of N98-4445A soy line (54%) which was not significantly different from that of 10-50 kDa fractions of the S03-543CR line (50 %) (Figure 5.1). The 10-50 kDa fractions from N98-4445A 4445A inhibited 73% of the HCT-116 cancer cells and this was statistically significant when compared to the positive control (77% inhibition). Figure 5.2 shows the activity of the peptide fractions on HCT-116 where <5kDa fraction from R95-1705 showed the only other significant inhibition. Even though the inhibition of Caco-2 cancer cells by the soy peptides was not statistically comparable to that of genistein, a 50% or more inhibition of the cancer cells is a significant amount of bioactivity against colon cancer growth. This significant inhibitory activity of the GI resistant hydrolysates against both cancer cell lines has potential use in restricting the malignancy of the disease.

Liver cancer cell growth inhibition (HepG-2)

The 10-50 kDa fractions of N98-4445A and S03-543CR soybean lines at a concentration of 800 µg/mL inhibited approximately 70% of HepG-2 cells which was not significantly

different from that of the positive control Genistein at 200 µg/mL (Figures 5.3). The <5 kDa fractions from N98-4445A line also showed significant inhibition of 64% which was similar to that of the positive control as per the statistical analysis. The inhibitory effect of the other peptide fractions was not statistically significant at a P value of <0.05 for the <5 kDa fractions from R95-1705.

Lung cancer cell growth inhibition (NCL-H1299)

On the NCL-H1299 lung cancer cells, the <5kDa fractions from N98-4445A and 5-10kDa fractions from S03-543CR showed 68% inhibition which was statistically comparable with the 73% inhibition of cell growth by the positive control Genistein. The 10-50kDa fractions from the high oleic acid soybean lines N98-4445A and S03-543CR also showed 50% and 47% inhibition of the lung cancer cells respectively, which was significantly lower than that of the smaller size fractions (Figure 5.4).

Dose response study on the inhibition of HCT-116 colon cancer cell growth

Even though the smaller peptide fractions had shown cell proliferation inhibition, the overall results from the study show that the 10-50kDa peptide fractions from the high oleic acid soybean lines N98-4445A and S03-543CR and had a significant inhibiting effect on both colon and liver cancer cell lines. A statistically significant inhibitory effect was demonstrated by the high oleic acid soybean peptide fractions although, dose used in the study were four times that of the positive control. Dose response study of the most effective (73% inhibition) 10-50kDa peptide fractions of N98-4445A soybean line showed that the inhibition of cancer cells was consistent at higher concentrations of 1000 µg/mL to 600 µg/mL on HCT-116 colon cancer cells and decreased to insignificant levels when the concentrations lowered from 500 µg/mL to 100 µg/mL (Figure 5.5). This test also showed that at the highest dosage level the cancer cell

inhibition was higher than that of the anticancer agent, Genistein, at 200 µg/mL. The minimum effective concentration of genistein (200 µg/mL) was used as a positive control for comparison that showed highest inhibitory activity. Beyond this dose, clumping of cells was observed, limiting effective counting of cells. Most effective inhibition of all the cancer cells tested occurred during 36 and 48 hours of incubation with the protein hydrolysates. The IC₅₀ value, which demonstrates the concentration of the hydrolysate where the cancer cell population is reduced by half, was 0.39 mg. The Analysis of Variance for the dose response on the HCT-116 colon cancer cells study showed an F ratio of 379.9 which was significant at <0.0001 ($R^2 = 0.99$) and the Student's T test value was 2.10 ($\alpha = 0.05$). The analysis also demonstrated that there was no significant difference in the activity between 400 µg and 500 µg doses while a 600 µg dose is the minimum inhibitory concentration required to achieve a significant inhibition of the colon cancer cell population.

Toxicity study of the most effective anticancer hydrolysates

The hydrolysates from all three soybean lines were tested for toxicity on normal human colon (CCD-18Co) and lung (LL 47 (MaDo)) cell lines. The MTS cell proliferation inhibition assay showed zero toxicity of the peptide hydrolysates which was comparable to negative control (media only) for both the cells.

Conclusion

Preparation of protein isolate from the meals helped in removal of extraneous matter including starch, and provided protein with high purity for hydrolysis with Alcalase. Gastro-intestinal resistance test showed that the peptide fractions can remain active when consumed. The high inhibition percentage of the high oleic acid lines could have possibly been due to the

amino acid make-up, sequence and the terminal residues of these smaller peptide fractions. The results were conclusive of the hypothesis that bioactive cancer inhibiting peptide fractions can be derived from enzymatic hydrolysis of the isolated soybean protein. The present study has significance since the peptides with cancer cell inhibiting activity can add value to oil separated meal from high oleic acid soybean lines. Cancer cell inhibiting peptides have been produced from high oleic acid soybean lines, N98-4445A and S03-543CR for the first time in this study with a possibility of generating substantial economic value to the soybean growers. It could be concluded that peptide fractions derived from meals of high oleic acid soybean lines have the property of inhibiting cancer cell growth in human cell lines and could have potential nutraceutical use against colon, liver and lung cancers.

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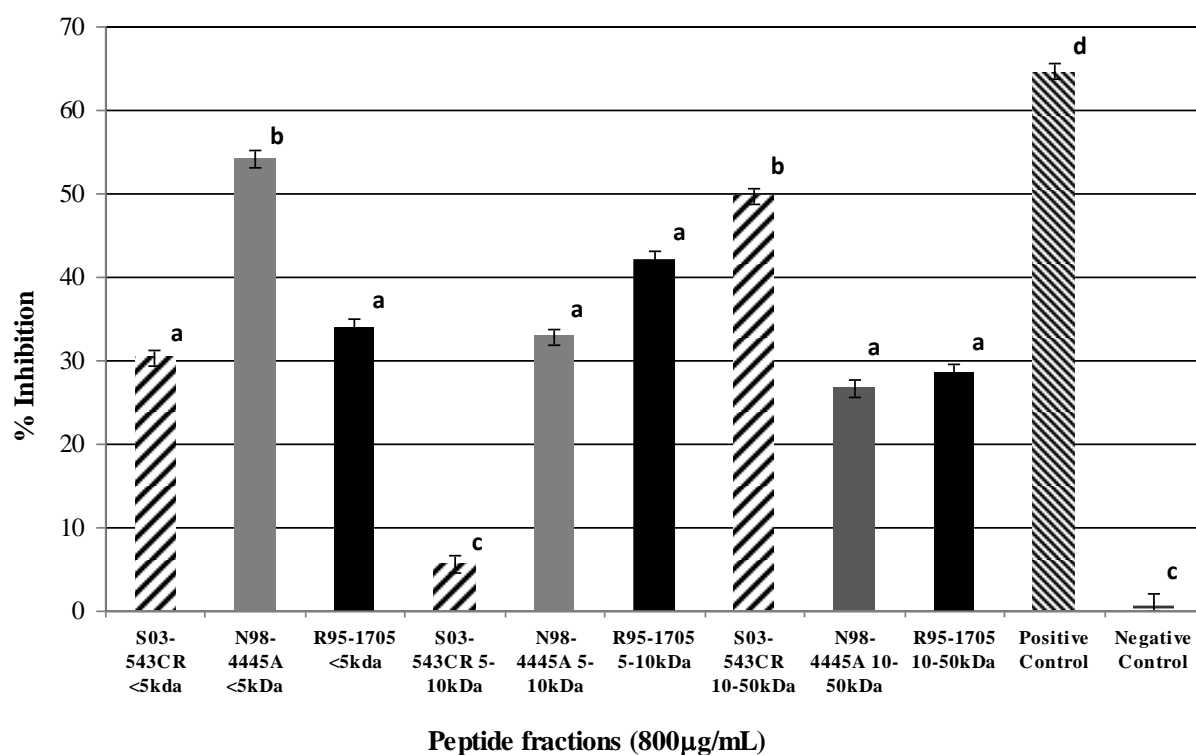


Figure 5.1. Caco-2 colon cancer cell line growth inhibition by gastro-intestinal resistant peptides from the three soybean lines. Positive control: Genistein 200 µg/ml, Negative control: Culture media only. Values (means \pm standard deviation) not connected with same letters are significantly different ($P < 0.05$). (Rayaprolu *et al.*, 2013).

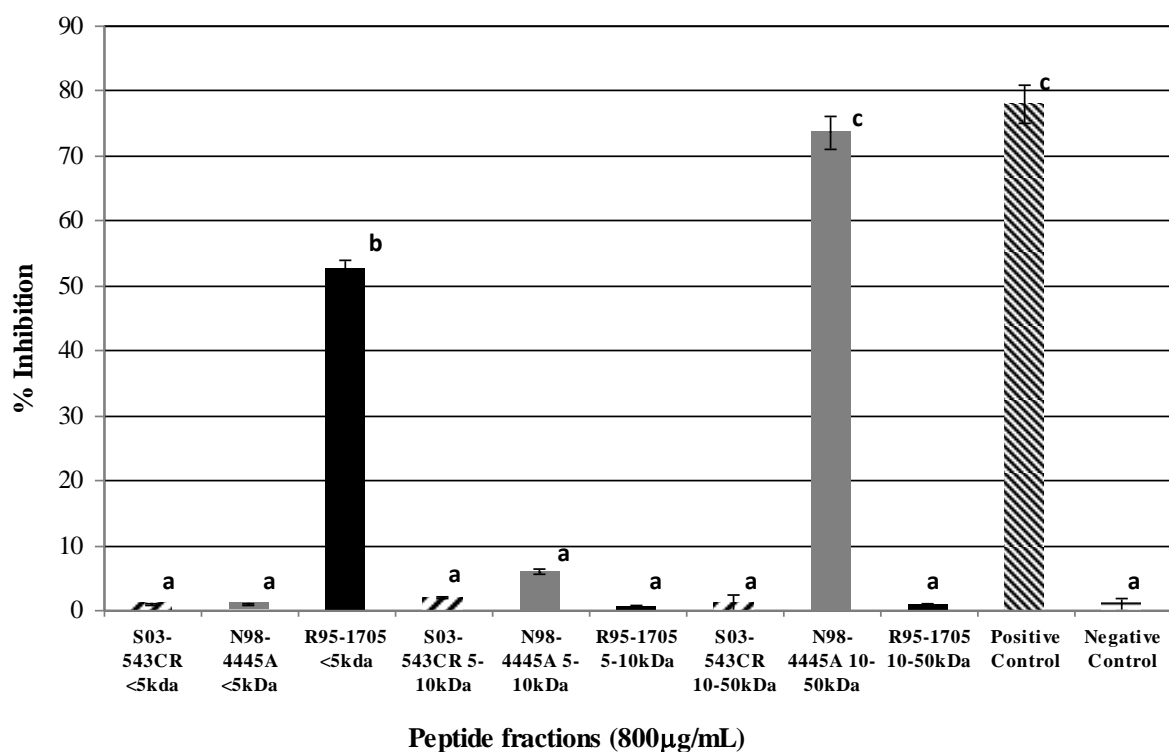


Figure 5.2. HCT-116 colon cancer cell line growth inhibition by GIPs from the three soybean lines. Positive control: Genistein 200 µg/ml, Negative control: Culture media only. Values (mean \pm standard deviation) not connected with same letters are significantly different ($P < 0.05$). (Rayaprolu *et al.*, 2013).

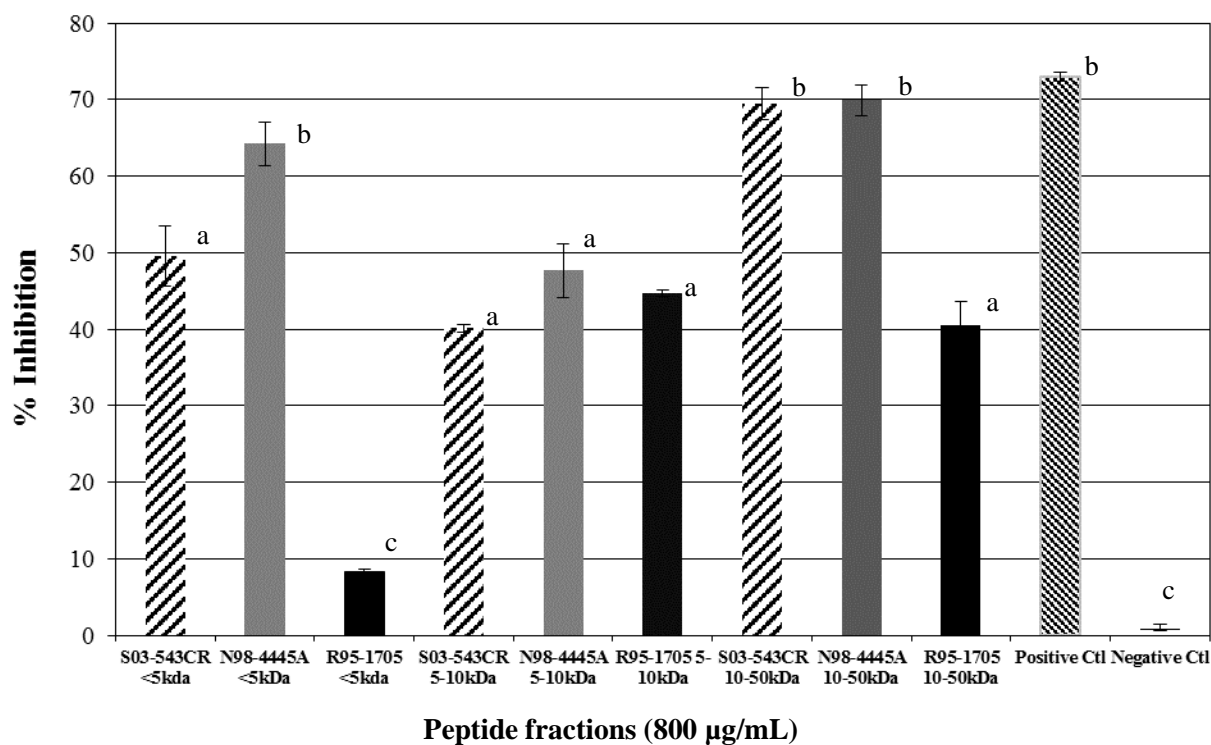


Figure 5.3. HepG-2 Liver cancer cell line growth inhibition by GIPs from the three soybean lines. Positive control: Genistein 200 µg/ml, Negative control: Culture media only. Values (means \pm standard deviation) not connected with same letters are significantly different ($P < 0.05$). (Rayaprolu *et al.*, 2013).

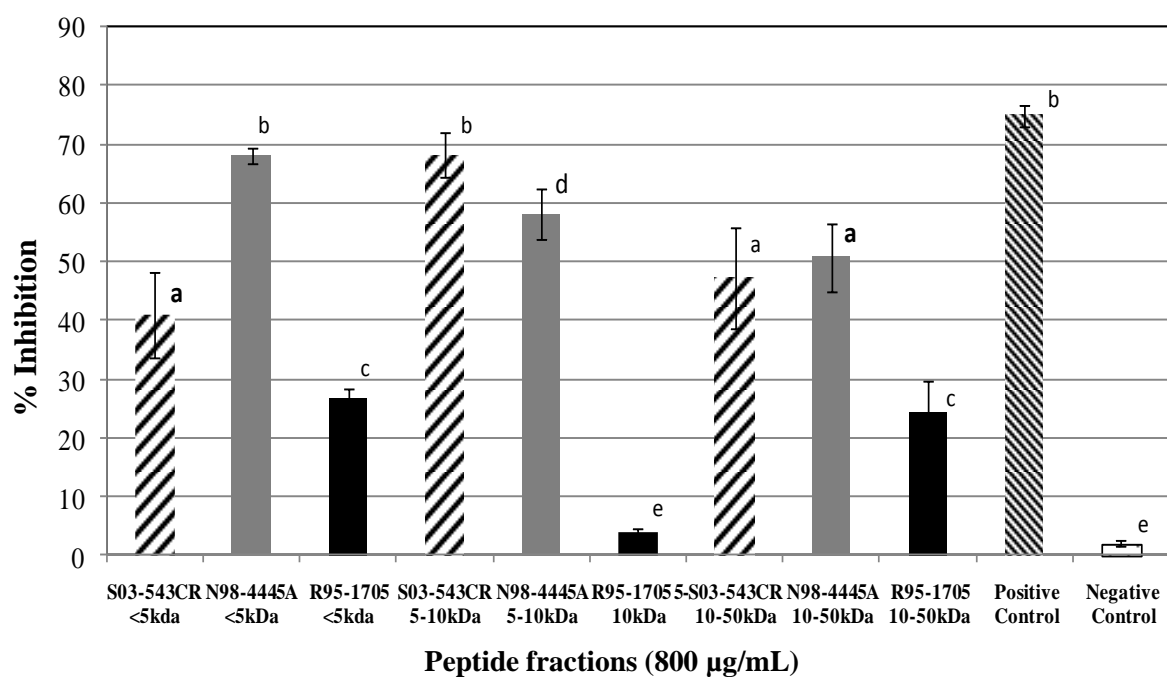


Figure 5.4. NCL-H1299 lung cancer cell line growth inhibition by gastro-intestinal resistant peptides from the three soybean lines. Positive control: Genistein 200 µg/ml, Negative control: Culture media only. Values (means ± standard deviation) not connected with same letters are significantly different ($P < 0.05$). (Rayaprolu *et al.*, 2013).

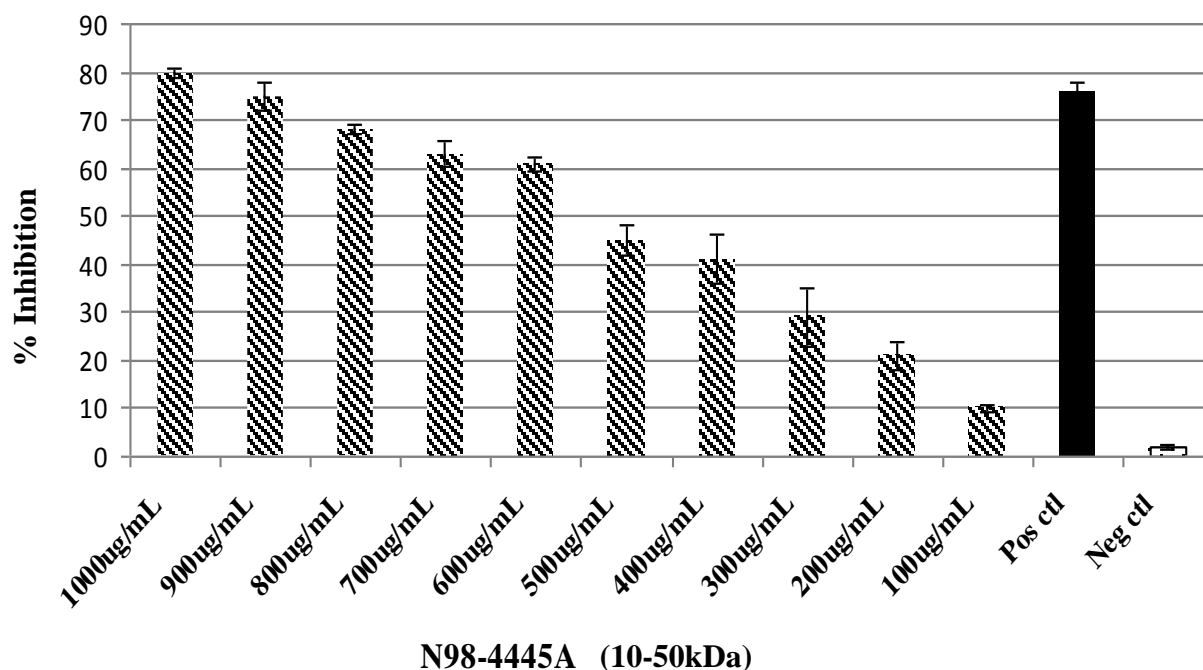


Figure 5.5. Dose response study (1000 μ g/mL to 100 μ g/mL) of 10-50 kDa peptide fractions of N98-4445A on HCT-116 colon cancer cell line. Pos ctl: positive control - Genistein 200 μ g/ml, Neg ctl: Negative control - Culture media only. Values (means \pm standard deviation) are in triplicate. (Rayaprolu *et al.*, 2013).

CHAPTER 6

Bioactivity of soybean protein fractions against human blood, breast and prostate cancer cell lines

ABSTRACT

In this study, we examined *in vitro* bio-activity of the peptide fractions against blood (CCRF-CEM and Kasumi-3), breast (MCF-7), and prostate (PC-3) cancer cell proliferation. Gastro-intestinal resistant peptide fractions (<5, 5-10 and 10-50kDa) prepared from seed proteins of two high oleic acid soybean lines - N98-4445A, S03-543CR and one high protein line - R95-1705, were tested for anticancer activity against human breast, blood and prostate cancer cell lines. Anti-proliferative cell titer assay was conducted to assess the inhibitory effects of the peptide fractions, while Trypan blue dye exclusion assay was used to determine the dose response of most effective fractions. Results showed that the peptide fractions inhibited the cancer cell lines up to 68% and the minimum inhibitory concentration ranged between 608-678 µg/mL. This multiple site *in vitro* cancer inhibition by GI friendly peptides could have potential use in food ingredients or nutritional supplements in alternative cancer therapy.

Introduction

The use of alternative methods in treating diseases like cancers has been on the rise in recent years. Conventional treatment methods like radiation and chemotherapy have become highly expensive and involve adverse side effects. Advanced cancer treatment methods employ a holistic approach by incorporating dietary or nutritional, spiritual and physical therapies along with drugs and radiation. As part of nutritional supplementation, bioactive peptides have a prominent role in alternative medicine which involves controlling diseases using food derived macro molecules (Zhang *et al.*, 1998, Korhonen & Pihlanto, 2003, Kannan *et al.*, 2008). Certain other bioactive molecules are from natural sources like plants (Ng 2003, Ohba *et al.*, 2004, Silva-Sánchez 2008). One of the major approaches to produce bioactive peptides was enzymatic hydrolysis or fermentation, especially from sources like soybean, wheat, corn, rice, sunflower, milk, eggs, and meat (Wang and de Mejia, 2005, De Mejia, 2006, Gibbs *et al.*, 2004).

Although it is used as a low cost animal feed, the potential nutritive nature, especially of the protein obtained from soybean meal components is well documented (Gallagher *et al.*, 2004). With studies conferring bioactivities to proteins, we found it necessary to test soybean protein hydrolysates for reducing the risk of cancer disease on cell culture models. Soybean meal has $\geq 45\%$ protein with possible unique amino acid types and sequence that could render bioactive nature by mitigating apoptosis in cancer cells (Hernandez-Ledesma *et al.*, 2009, Hsieh *et al.*, 2010). It is also of significant importance for the bioactive peptides to possess resistance to gastrointestinal environment when ingested. Soy has several value-added uses and has proven to be a source of peptides derived by enzymatic hydrolysis that have shown nutraceutical and functional properties (Rayaprolu *et al.*, 2013). However, studies are lacking on the effect of peptides from high oleic acid lines on cancers like blood (leukemia), breast and prostate, which

are estimated to cause a total of 94,000 deaths (2014) in the United States (ACS 2014). This is the first study that utilized high oleic acid soy meal as a starting raw material to produce peptides with anticancer activity against breast, blood and prostate cancers that can have a significant impact in their treatment. Our previous studies have shown anticancer activities against colon, liver and lung cancer cells (Rayaprolu *et al.*, 2013). The present study is in continuation to evaluate anti-proliferative activity against blood (CCRF-CEM, Kasumi-3), breast (MCF-7) and prostate (PC-3) cancer cell lines. Utilization of a low cost soybean meal to produce effective anticancer peptides is the significant achievement of this research. The objectives of this research were to select two high oleic acid and one high protein soybean lines to prepare peptides, prepare protein hydrolysates with enzyme Alcalase under optimum conditions, treat peptides with simulated gastro-intestinal juices, fractionate the peptides using ultrafiltration and investigate the anticancer activity of GI resistant fractions on the human blood, breast and prostate cancer cell lines.

Materials and Methods

Seeds from three soybean lines, S03-543CR and N98-4445A (high in oleic acid) and R95-1705 (high in protein, non-transgenic) were provided by Dr. Pengyin Chen (Professor, Plant Sciences Department, University of Arkansas). Food grade enzyme, Alcalase, was purchased from Novozyme Inc. (Bagsvaerd, Denmark) and gastro-intestinal enzymes pepsin and pancreatin were purchased from Sigma-Aldrich (St. Louis, MO). The ultrafiltration system was purchased from Koch membrane systems (Massachusetts, USA). The cell lines and media were obtained from ATCC (Manassas, VA) and Fetal Bovine Serum was purchased from Atlanta Biologicals (Atlanta, GA, USA). The reagents for cell proliferation inhibition were purchased from Sigma-

Aldrich (St. Louis, MO) and Promega Inc. (Madison, WI). The cells were observed for morphological changes using a Vistavision inverted phase contrast microscope (VWR, West Chester, PA). All other chemicals and supplies were obtained from VWR (West Chester, PA). Inverted phase contrast microscope was purchased from (VWR Inc., West Chester, PA).

Preparation of Enzyme-Treated Soy Protein Hydrolysates.

The soybean seeds were ground to flour and sieved (250 μ m) for uniform particle size. The lipid was removed using the solvent, N-hexane, to collect the residue which is called the soybean meal. The meals were suspended in water (10% w/v) and protein was isolated at an alkaline pH of 9.5 under ambient temperature conditions. The protein was separated from solution using isoelectric pH (4.5) as described previously (Rayaprolu *et al.*, 2013) and the isolates obtained with ~90% purity were then freeze dried and stored at 5 $^{\circ}$ C.

The protein isolates were suspended in water (10% w/v), mixed to homogeneity, the pH was adjusted to 7.0 and incubated at 55 $^{\circ}$ C. These isolates were treated with the enzyme Alcalase (at a concentration of 1.675 Anson units) for 1 hour based on the optimal conditions for a 30% degree of hydrolysis using the Box-Behnken Design of the Response Surface Methodology (Diniz & Martin, 1996, Rayaprolu *et al.*, 2013). The enzyme was inactivated at a temperature of 90 $^{\circ}$ C, and hydrolysates were separated by centrifugation, freeze dried and stored at 5 $^{\circ}$ C.

Preparation of Gastrointestinal (GI) Juice Resistant Soy Peptide Fractions.

The enzyme treated hydrolysates were passed through a simulated gastric and intestinal solution as previously described (Rayaprolu *et al.*, 2013). The GI-resistant protein hydrolysates were fractionated using Ultrafiltration system equipped with 1" diameter hollow-fiber polysulfone membrane cartridges. Nominal molecular weight cut-offs (MWCO) of 50, 10, and 5 kDa sequential ultrafiltration columns were used. The filtered soluble GI-resistant protein

hydrolysates were passed through the columns as the retentates and permeates were collected separately for each soybean line. Approximately a quarter of the total volume of protein hydrolysates were obtained which are now addressed as GI-resistant peptide fractions (<5kDa, 5-10kDa and 10-50kDa). The nine molecular cut-off fractions from the three soybean lines (3 soybean lines X 3 fractions) were freeze-dried and were tested for inhibitory activity against human blood (CCRF-CEM and Kasumi-3) and breast (MCF-7) and prostate (PC-3) cancer cell lines.

Propagation of Human Cancer Cell Lines.

Human prostate cancer cell line PC-3 (ATCC# CRL-1435) was cultured in F-12K medium, Kasumi-3 (ATCC# CCL-2725) and CCRF-CEM (ATCC# CCL-119) blood cancer cell lines were cultured in RPMI-1640 medium, and the breast (epithelial) cancer cell line MCF-7 (ATCC# HTB-22) was cultured in Eagle's Minimum Essential Medium. The peripheral blood cells (ATCC# PCS-800-010) were cultured in the recommended Hank's Balanced Salt Solution and used for toxicity tests. All the media were devoid of antibiotics and supplemented with 10% fetal bovine serum. The cells were incubated at 37 °C Cells in a humidified atmosphere containing 5% carbon dioxide. The growth phase of all cancer cells was monitored using an inverted phase contrast microscope. After cell growth achieved 80%, they were treated with the peptide fractions, <5kDa, 5-10kDa and 10-50kDa, from the three soybean lines. The anti-proliferation assay was performed to determine the anticancer activity.

Determination of Anti-Proliferative Activity of the Peptides - MTS Colorimetric Assay.

The [3-(4,5- dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS)-based cell titer assay was used to determine the effect of soybean peptide fractions on the cytotoxicity against cancer cells.. The blood cancer cell lines, CCRF-CEM and Kasumi-3, were allowed to

grow in suspension (medium) since they do not attach to the well surface. Fresh growth media was added by separating the cells using centrifugation (1000 x g for 5 min). The pellet was rinsed and re-suspended in fresh media before transferring into the 96-well plate for 36 hour incubation at 37 °C and 5% CO₂. The prostate (PC-3) and breast (MCF-7) cancer cells were cultured in the 25 cm³ flask with the growth media to form a monolayer on the flask surface. The cells were checked for conformation (Trypan blue dye exclusion test) for minimum counts of ≥ 3000 . They are detached from the flask surface by adding Trypsin-EDTA and re-suspended in respective fresh media. The four types of cancer cells were transferred to separate 96-well plates (200 μ L/well) for a 36 hour incubation at 37 °C and 5% CO₂. The nine soybean peptide fractions dissolved in water (800 μ g/mL) were added to the wells with the cancer cells. A column of wells were treated with saline (negative control) while Genistein (200 μ g/mL) was used as positive control for comparison of growth inhibition. The cancer cells were incubated for 18-36 hours while being observed for cell death due to the test compounds. The MTS dye was added to determine cell survival and termination was observed by the formation of colored formazan product. The 96-well plate was read at 490 nm in a Microplate Reader (Bio-Rad Inc., Hercules, CA, USA) and the percent inhibition was determined based on the absorbance value (Rayaprolu *et al.*, 2013).

Quantitative Evaluation of Dose Response by Trypan Blue Dye Exclusion Assay.

The cancer cell growth inhibition based on dosage of GI resistant soy peptide fractions was established based on the Trypan Blue dye exclusion assay. This is conducted for the GI-resistant peptide fractions that showed highest cytotoxicity against the four cancer cells tested. For trypan blue staining, 500 μ L of cells from each cancer cell culture were aseptically transferred to a 24 well plate and incubated for 72 hours. The peptide fractions with

concentrations ranging from 200 to 1000 µg/mL were added to the wells along with media and incubated for 48 to 60 hours at 37 °C and 5% CO₂. The wells were treated with 0.4% (w/v) Trypan blue solution prepared in 0.81% NaCl and 0.06% (w/v) dibasic potassium phosphate. Cell counts were determined using a dual-chamber hemocytometer and a phase contrast microscope. Numbers of viable cells were recorded based on the stain retention.

Calculations and Statistics.

All tests were conducted in triplicate to minimize experimental error and were repeated for reproducibility. The JMP 12.0 statistical software (SAS institute, Cary, NC) was used for all data analyses.

Results and Discussion

Protein hydrolysate preparation

Protein isolates prepared from the meal (defatted flour) of the R95-1705, S03-543CR and N98-4445A soybean lines had a purity of 90% or higher due to the effective alkaline extraction procedure (Rayaprolu *et al.*, 2013). Alcalase, a food grade enzyme, was used to produce protein hydrolysates from the isolates. The degree of hydrolysis was used as the response variable in the modelling of enzymatic hydrolysis and varying molecular sized protein fragments were collected (Nielsen *et al.*, 2001). This was in congruence with the premise of this study which is to determine the bioactivities of peptides with various lengths and amino acid sequences. The simulated GI resistance test demonstrated that the peptides are resistant to digestion in the GI tract and will remain bioactive without undergoing any degradation when consumed. The protein content of the GI-resistant fractions from the three soybean lines ranged between 85 and 88% purity.

Anti-proliferative activity of GI-resistant peptide fractions against cancer cell lines

Previous studies have shown that protein products are effective against various cancers (Bylund *et al.*, 2000, Badger *et al.*, 2005). All the 4 cancer cell types had counts ranging from 3000 to 5000 (>70% confluence) during the growth phase in the culture flasks. The morphological confirmation of the blood, prostate and breast cancer cells during initial proliferation showed no shrinkage or nuclear blebbing, which was essential for the experiment.

The results showed that 68% of CCRF-CEM blood cancer cells were inhibited by the 10-50kDa fraction from the N98-4445A soybean line (Figure 6.1) which was the highest among all fractions tested. The 5-10kDa fraction from the same soybean line showed 52% cytotoxicity which was significantly different from the 5-10kDa fraction of S03-543CR (41% inhibition). On the Kasumi-3 blood cancer cell line <5kDa (52%) and 10-50kDa (49%) fractions from the N98-4445A soybean line and 5-10kDa (55%) fraction from S03-543CR inhibited the cell growth with no statistically significant difference ($P < 0.05$) amongst them (Figure 6.3). However, the 5-10kDa of S03-543CR had the highest inhibition among all the fractions. The 5-10kDa fractions from S03-543CR and R95-1705 soybean lines showed 41 (CCRF-CEM) and 37 (Kasumi-3) percent inhibition respectively which was the only other significant reduction observed during the MTS assay on the two blood cancer cell lines (Figures 6.1 and 6.2).

The 5-10kDa fraction from S03-543CR soybean line showed 63% inhibition against the PC-3 prostate cancer cells, while the 10-50kDa fraction from N98-4445A soybean line inhibited 58% of the cells (Figure 6.3). Similar sized fraction (5-10kDa) obtained from R95-1705 soybean line also showed 38% inhibition of the PC-3 cells but there was significantly lower. The other fractions that were tested did not demonstrate any activity against the prostate cancer

cells. This is the first study that revealed the efficiency of GI resistant fractions from a seed protein in inhibiting *in vitro* prostate cancer cell proliferation.

The MCF-7 breast cancer cells were used as a model system for testing anti proliferative activity and biochemical pathways by various researchers (Doyle *et al.*, 1998, Kannan *et al.*, 2009, Chen *et al.*, 2012 and Li *et al.*, 2014a). The 5-10kDa fraction from the R95-1705 soybean line showed a significantly high activity among all the fractions with 63% inhibition of MCF-7 cells (Figure 6.4). This was not statistically different (P value <0.05) from the positive control. This result has a major impact on future studies involving anticancer agents from plant sources since R95-1705 is a non-transgenic (non-GMO) soybean line. The <5kDa and 10-50kDa from the R95-1705 soy line also inhibited the MCF-7 cells and the activity was significant in comparison to the other fractions. The three fractions from the S03-543CR high oleic acid line showed extremely poor activity against the breast cancer cells while the fractions from N98-4445A soybean line showed 18-30% inhibition of the MCF-7 cells.

The normal peripheral blood cells were required to be cultured immediately after thawing the procured frozen stocks and showed 2×10^3 counts when cultured with the growth medium in the 96-well plates. The toxicity test of the peptide fractions on normal human blood cell line showed no significant effect (< 2-5%) in comparison to Genistein that showed inhibition of normal blood cells by 11%. These results demonstrate that the peptide fractions can reduce the cancer cell growth without affecting the neighboring normal human cells. A summary of effective cancer cell inhibitory activity demonstrated by the various peptide fractions is given in Table 6.1. Further purification of the fractions into pure peptides may provide enhanced anticancer effect. Other researchers have demonstrated an effective anticancer activity of single peptides against breast cancer (Kannan *et al.*, 2009 and Li *et al.*, 2014a,

2014b). These results have demonstrated the immense potential of peptide fractions and peptides in reducing in vitro breast cancer cell proliferation. Continuation of this research with clinical trials may provide potential drug-free or supplemental therapeutic solutions in treating cancers.

Dose response study based on Trypan blue dye exclusion method

The Trypan blue dye exclusion test has been used for numerically quantifying the cell counts. This test provided the cell counts against the dose response of the specific fractions that showed highest inhibitory activity based on MTS colorimetric method. The established cell counts based on Trypan blue assay provided a quantitative scrutiny of the effectiveness of peptide fractions against the cancer cells. The results from the assay correspond to the cell survival/viability percentage calculated based on the counts in comparison to a known positive control, Genistein. Among the fractions, the 5-10kDa fraction from S03-543CR soybean line showed the best growth inhibition of both prostate and Kasumi-3 blood cancer cell lines (Figure 6.5). The dose response showed significant increase in activity at $>600 \mu\text{g/mL}$ which was observed on PC-3, CCRF-CEM and MCF-7 cell lines with approximately 50% reduction in cell survival. The 5-10kDa fraction from the S03-543CR soy line showed the highest reduction at $1000 \mu\text{g/mL}$ on PC-3 cell counts which was not significantly different in comparison to Genistein (P value <0.05). Although the cell survival % reduced to less than 20% in the blood cancer cell lines (CCRF-CEM and Kasumi-3) the positive control had the significantly higher inhibition. On the MCF-7 breast cancer cell line, the 5-10kDa fraction showed a significant reduction in cell survival at the $\geq 800 \mu\text{g/mL}$ dose. The minimum effective concentration (EC_{50}) for the 5-10kDa (S03-543CR) fraction against PC-3 prostate cancer cells was found to be $608 \mu\text{g/mL}$. The 10-50kDa fraction from N98-4445A against CCRF-CEM blood cancer cells was $678 \mu\text{g/mL}$ while

the 5-10kDa fraction of S03-543CR had an EC₅₀ value of 611 µg/mL on the Kaumi-3 cells. On the MCF-7 breast cancer cell line, the 5-10kDa fraction from R95-1705 showed a significant reduction in cell survival (10%) at a dosage of 1000 µg/mL dose with an EC₅₀ of 654 µg/mL.

Conclusion

Enzymatic hydrolysis (with and without protein extraction) has been proven to be a promising approach to obtain peptides from protein sources that are considered a bio-waste (Kannan *et al.*, 2008, 2009 and Rayaprolu *et al.*, 2013). This study is the first of its kind to report utilization of soybean meal to obtain bioactive peptides against multiple-site cancer cells. Soy peptide fractions obtained after proteolytic digestion and tested for GI resistance showed potent anticancer activity against human blood, breast and prostate cells. These GI resistant peptides are suitable for use in food product applications due to potential bio-availability when consumed.

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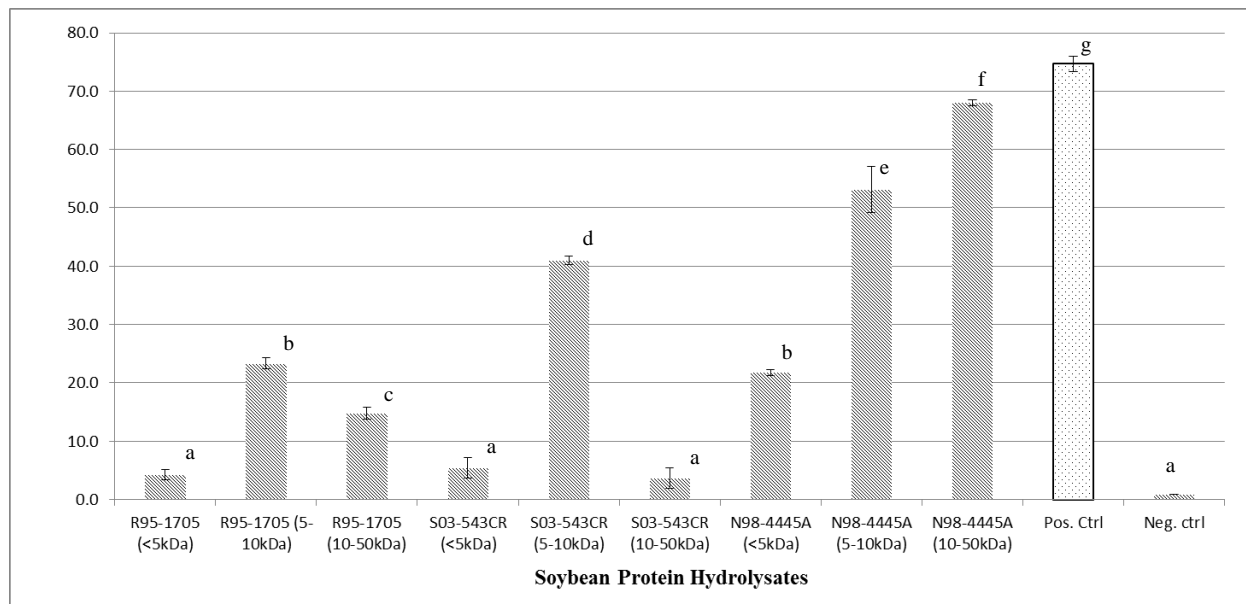


Figure 6.1. Bioactivity of peptide hydrolysates prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against CCRF-CEM human blood cancer cells. Positive control: Genistein 200 µg/ml, Negative control: Saline. The bars represent % inhibition values expressed as means \pm standard deviation (of triplicates) and those not connected by same letter are significantly different ($P < 0.05$).

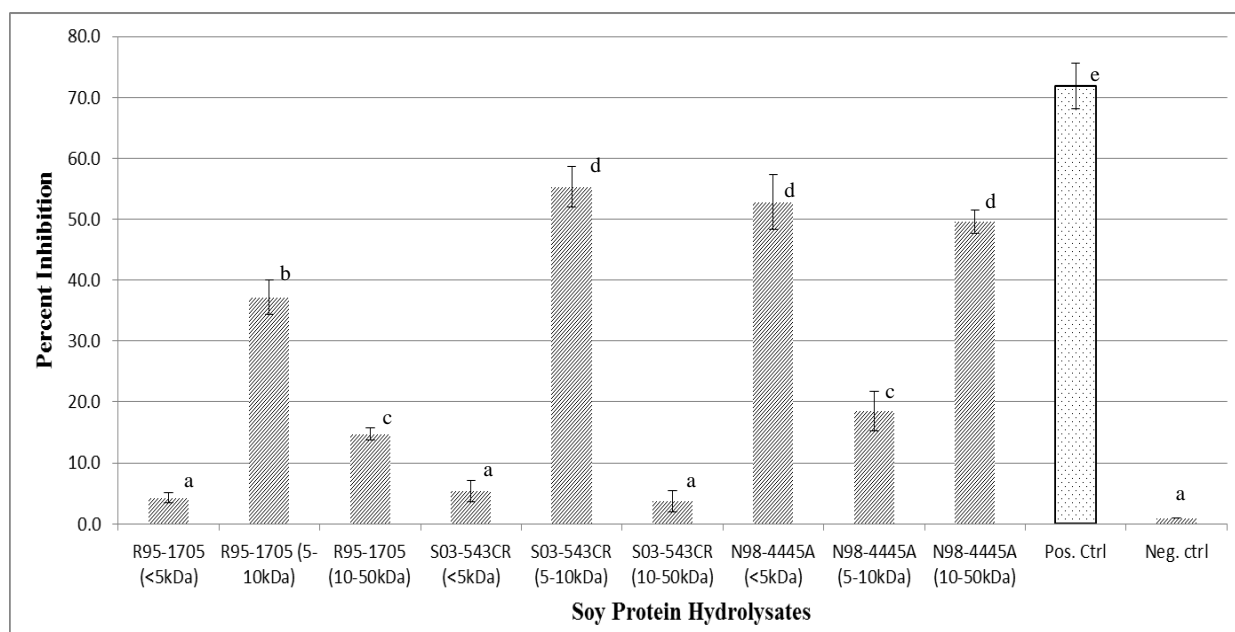


Figure 6.2. Bioactivity of peptide hydrolysates prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against Kasumi-3 human blood cancer cells. Positive control: Genistein 200 μ g/ml, Negative control: Saline. The bars represent % inhibition values expressed as means \pm standard deviation (of triplicates) and those not connected by same letter are significantly different ($P < 0.05$).

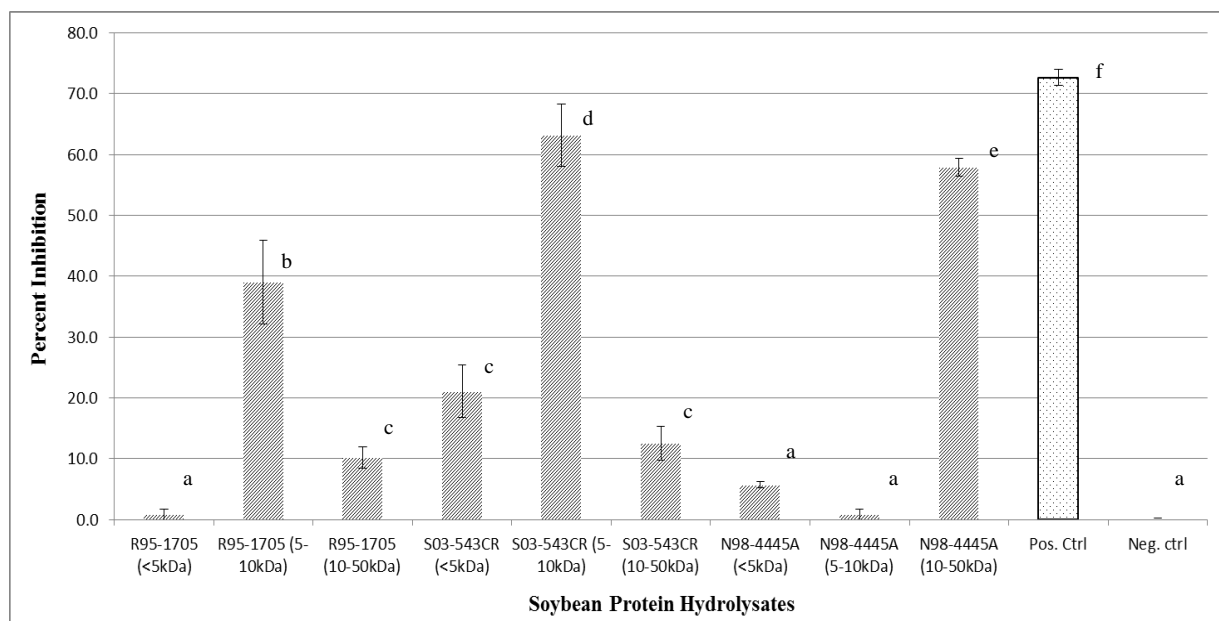


Figure 6.3. Bioactivity of peptide hydrolysates prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against PC-3 human prostate cancer cells. Positive control: Genistein 200 µg/ml, Negative control: Saline. The bars represent % inhibition values expressed as means ± standard deviation (of triplicates) and those not connected by same letter are significantly different (P < 0.05).

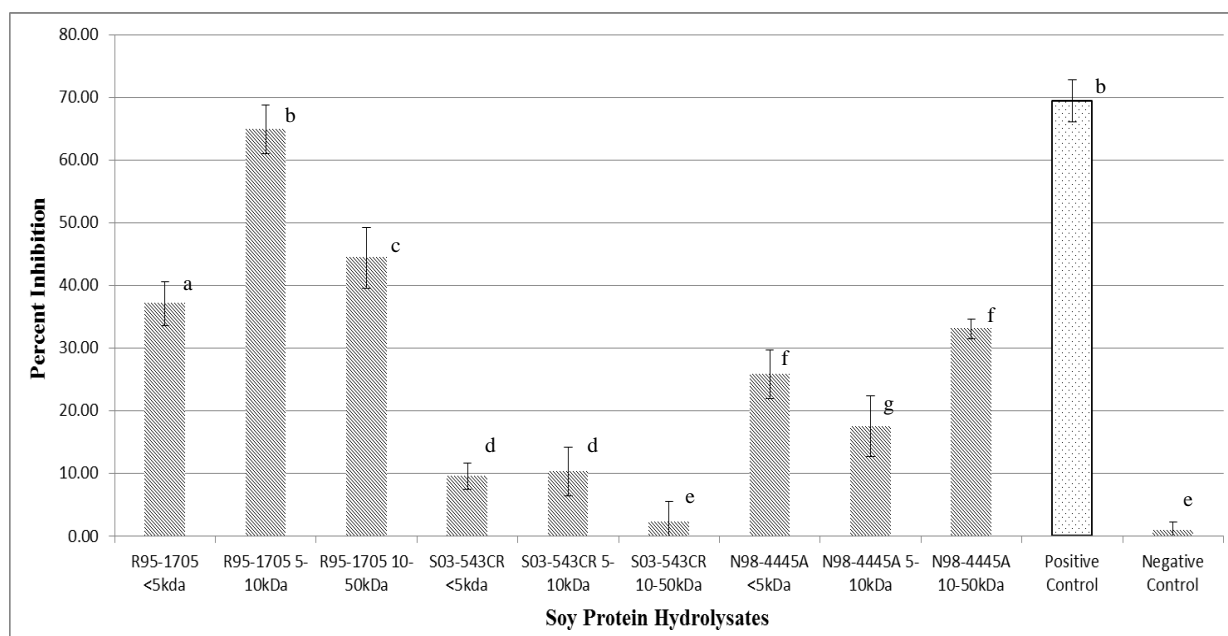


Figure 6.4. Bioactivity of peptide hydrolysates prepared from two high oleic (N98-4445A and S03-543CR) and one high protein (R95-1705) soybean lines against MCF-7 human breast cancer cells. Positive control: Genistein 200 µg/ml, Negative control: Saline. The bars represent % inhibition values expressed as means ± standard deviation (of triplicates) and those not connected by same letter are significantly different ($P < 0.05$).

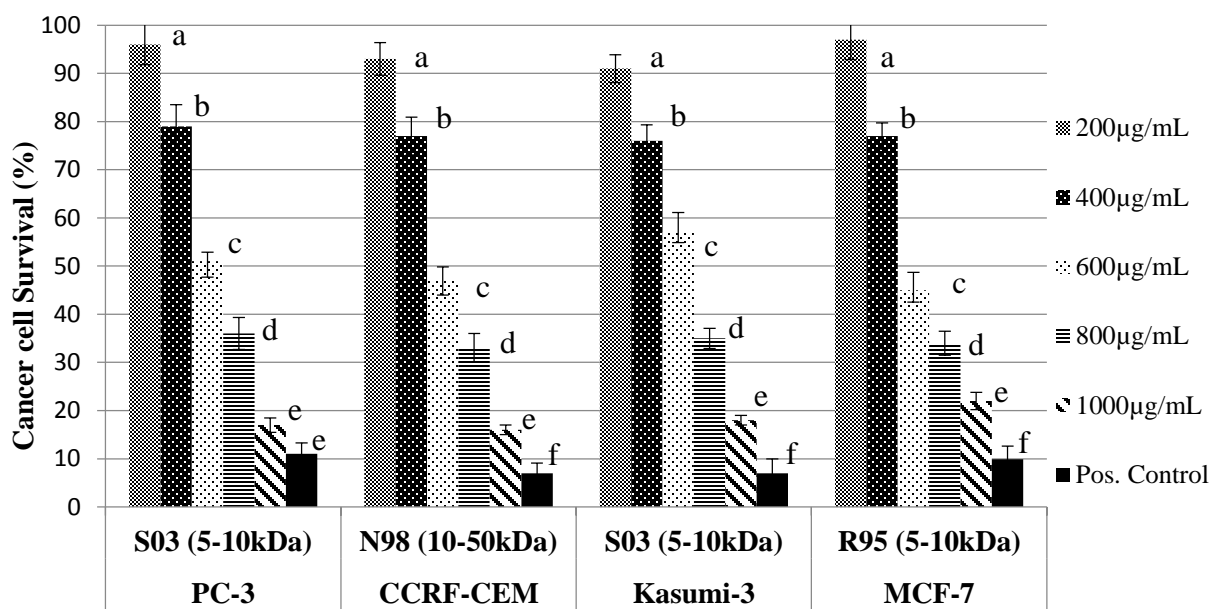


Figure 6.5. Dose response study of GI resistant fractions with highest anticancer activity based on Trypan blue dye exclusion assay. Positive control: Genistein 200µg/ml. PC-3: Prostate cancer; CCRF_CEM: Blood cancer; Kasumi-3: Blood cancer; and MCF-7: Breast cancer. Soybean lines are designated as: S03 = S03-543CR, N98 = N98-4445A and R95 = R95-1705. Values expressed as means \pm standard deviation (of triplicates) and those not connected by same letter for each cancer type and protein hydrolysate combination are significantly different ($P < 0.05$).

Table 6.1. Summary of anticancer activity of selected peptide fractions prepared from three soybean lines against human cancer cells.

Type of Cancer cell	<i>Anticancer activity (% inhibition) of GI-resistant peptide fractions</i>		
	<u>(N98) 10-50kDa</u>	<u>(S03) 5-10kDa</u>	<u>(R95) 5-10kDa</u>
Blood, CCRF-CEM	68.0±0.5 ^a	41.0±0.7 ^b	23.3±0.9 ^c
Blood, Kasumi-3	49.6±1.9 ^b	55.3±3.3 ^a	37.2±2.7 ^c
Breast, MCF-7	33.1±1.6 ^b	10.4±3.9 ^c	64.9±3.9 ^a
Prostate, PC-3	57.9±1.7 ^b	63.2±5.1 ^a	39.0±6.8 ^c

The values presented are means ± standard deviations from triplicate analysis. The values not connected by same letters in each horizontal row are significantly different (P <0.05).

CHAPTER 7

Purification and characterization of single peptides with anticancer activities

Abstract

The objective of this research was to identify and purify peptides from the protein hydrolysate fraction prepared from soybean line that previously showed significant activity against human cancer cell lines. Protein isolate from a high oleic acid soybean line N98-4445A was used to prepare the peptide pools. The 10-50kDa molecular size cut-off fractions showed the highest activity which were purified using peptide specific affinity chromatography column. Reverse phase HPLC identified three peptides from the fractions. These three individual peptides were tested for anti-proliferative activity against HCT-116 colon cancer cell line, CCRF-CEM blood cancer cell line and HepG-2 liver cancer cell line using (3-(4, 5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell titer assay. Enhanced colon cancer cell inhibition activity of 80% was observed after testing a pure peptide which also demonstrated a time-response of 96 hour after incubation based on Trypan blue dye exclusion study. This single peptide with highest activity had a molecular size of 18kDa with 158 amino acid residues. The impact of this study lies in deriving a pure single peptide with anti-proliferative activity on human colon and blood cancer cells.

Introduction

Physical and chemical treatments, including enzymatic catalysis of food proteins are conducted to derive bioactive peptides. But, chemical treatments may contribute to inactive peptides by preventing them from being released from the parent protein – as resistant peptide bonds can be generated (Rutherford-Markwick and Moughan, 2005). However, enzymatic hydrolysis has been proven to be the most effective method to derive bioactive peptides from protein sources (Seppo *et al.*, 2003, Korhonen and Pihlanto, 2006, Kannan *et al.*, 2008, 2009 and 2012, Rayaprolu *et al.*, 2013 and 2015). Controlled enzymatic hydrolysis can generate peptides that can elicit bioactivity including anti-proliferative action against cancer cell lines. Studies have shown that enzyme catalysis provides peptides from various food sources that can act as physiological biomolecules (Yoo *et al.*, 1997, Ng *et al.*, 2003, Korhonen and Pihlanto, 2003, Ohba *et al.*, 2004, Gibbs *et al.*, 2004, Kannan *et al.*, 2010, Li *et al.*, 2014). Identifying the peptide structure and the amino acid sequence can provide further details into its functional properties and its synthesis for pharmaceutical and nutraceutical applications.

Proper analytical separation and purification techniques can identify peptides that are important biological modulators. Various analytical separation techniques include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Deeslie and Cherian (2006) studied soy protein hydrolysate fractionation using ultrafiltration membranes using membrane pore sizes that were specific to the molecular size distribution of the peptides with functional properties. Reverse phase high performance liquid chromatography (RP-HPLC) with ion-exchange or size exclusion chromatography has been widely used for efficient purification of the peptides (Le´onil *et al.*, 2000) prepared using catalysis with Alcalase and flavourzyme (Prakash *et al.*, 2002). A peptide inhibiting obesity was isolated and purified from soybean protein

hydrolysate using a systematic separation with ultrafiltration, gel filtration chromatography, and reverse-phase HPLC by previous researchers (Kim *et al.*, 2007). These methods provided evidence to distinguish and select the appropriate search results for peptide purification.

For a comprehensive characterization of complex mixtures of peptide, chromatographic analytical methods were used to better separate peptides prior to mass spectrometric analysis (Léonil *et al.*, 2000). These methods can be used in succession to achieve a very high degree of resolution. The most effective way to identify and quantify proteins and their modifications by mass spectrometry (MS) is to analyze enzyme (trypsin) digests of the proteins of interest. Liquid chromatography (LC) coupled with mass spectrometry is by far the most informative technique for determining the purity of protein/peptide samples (Ono *et al.*, 2006, Peng *et al.*, 2003). It is a technique of analytical chemistry to identify the molecular mass of isolated individual peptides present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions. The mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio which provides the number of ionic components present and indicates the length of the proteins/peptides (Desiderio, 1990, Sunner *et al.*, 1995). Database search when used in conjunction with MS offers increasingly enhanced information in the characterization of peptides. Of the ionization modes, Matrix assisted laser desorption ionization (MALDI) has made it possible to not only determine the accurate mass of peptides obtained in a hydrolysate of food proteins but also is able to identify the purity of a pure protein or peptide, determine its fragmenting patterns and also determine the de novo sequence of the amino acids.

Our previous studies have shown that a 10-50kDa fraction obtained from a high oleic acid soybean line has anti-proliferative activity against blood (CCRF-CEM), colon (Caco-2 and HCT-116) and liver (HepG-2) cancer cell lines. Hence, the objective of this study was to

separate single pure peptides from a pool using RP-HPLC and size exclusion, and test them on colon, blood and liver cancer cell lines for investigation of cell proliferation inhibitory properties. Further characterization and amino acid sequence of the single peptide, that showed highest cancer cell cytotoxicity, was determined by mass spectrometry.

Materials and methods

Purification of hydrolysates by HPLC to separate single peptides

Soy protein isolate was prepared by alkaline hydrolysis (pH 9.5) from the seeds of N98-4445A soybean line. It was hydrolyzed using Alcalase enzyme, tested for simulated gastrointestinal juice resistance and fractionated using ultrafiltration columns with definite molecular size cut-offs (Rayaprolu *et al.*, 2013). The 10-50kDa peptide fractions so derived showed highest anti-proliferation activity against colon, liver and blood cancer cells. This fraction was selected for further purification using a Supelco Bio Wide pore C-18 semi-preparative chromatography column from Sigma Aldrich (St. Louis, MO) connected to an HP 1050 series HPLC system from Agilent Technologies (Santa Clara, CA), to identify and separate individual peptides.

A two solvent reverse phase HPLC analysis of the peptide fractions was conducted. Solvent A: 0.1 % Trifluoro Acetic acid in 10mM Phosphate buffer at pH 7.3 and solvent B: 50 % Acetonitrile in water with 0.1 % Trifluoro Acetic acid in a linear gradient for a 90 minute run. The flow rate was maintained at 1 mL/min with the solvent A: 50 % to 0 % for 50 min and solvent B: 0 to 100 % for 90 min.

Size exclusion chromatography of peptides

Analytical grade Sephadex G-15 hydrophilic resin was purchased from Sigma Aldrich (St. Louis, MO) and packed into a glass column and equilibrated with Millipore water. The

column was connected to a semi-preparative HPLC system with one pump running in a binary phase. A 1mL sample of 10-50kDa peptide hydrolysate (~0.5 mg/mL protein concentration) was loaded onto the column at 1 mL/min flow rate. Five bed volumes of degassed, double filtered water were used to wash the peptide fractions as they pass through the resin beads in the stacked column. The individual peptides from the fraction were eluted using Millipore water with a run time of 85 minutes. The eluates were collected, concentrated in the Spin-X concentrator from Corning life sciences (Corning, NY), lyophilized and stored at 4 °C until used for cancer cell proliferation inhibition activity. The bioactivity against cancer cells by the pure peptides obtained after ion-exchange was determined using the cell proliferation inhibition assay.

Determination of anti-proliferation activity against cancer cells by the pure peptides.

The [3-(4,5- dimethyl thiazole-2-yl)]-2,5-diphenyl tetrazolium bromide (MTS) based cell titer assay was used to determine the effect of soybean peptide fractions on the cytotoxicity against cancer cells.. The cancer cell lines CCRF-CEM (blood), HCT-116 (colon) and HepG-2 (liver) were cultured in the required growth media in in the 25 cm³ flasks. The blood cancer cells remained suspended over the length of the culture and did not attach to the well surface. Hence, fresh growth media was added every 72 hours by separating the cells using centrifugation (150 g for 5 min). The colon and liver cancer cell lines were detached from the flask surface by adding Trypsin-EDTA and re-suspended in respective fresh media. All cell lines were incubated at 37 °C and 5% CO₂ for 72 hours and checked for confluence using Trypan blue dye exclusion assay (Kannan *et al.*, 2008). After ensuring a minimum count of ≥ 3000 they were transferred into separate 96-well plates. The three peptides purified from the 10-50kDa fraction obtained from N98-4445A soybean line were added (dissolved in water) to the wells with the cancer cells. Saline (1%) and genistein (200µg/mL) were used as controls for comparison of growth

inhibition. The incubated 96 well plates were observed periodically for cell death due to the test compounds. The MTS dye was added to the wells to determine mitochondrial activity and termination of the assay was observed due to the formation of colored formazan product. Absorbance of the well constituents were measured at 490 nm using a micro Epoch plate reader from Biotek (Winooski, VT) and the percent inhibition was determined based on the absorbance value.

Time response study of most effective peptide on colon cancer cells

The Trypan blue dye exclusion assay was used to determine the time response of the most effective peptide for cytotoxicity against the blood and colon cancer cells over the incubation period (Strober, 2001). The cells were cultured in 25 cm³ flasks for $\geq 70\%$ confluence and transferred to a 24-well plate. The peptide (700 $\mu\text{g/mL}$) was added to the cells and incubated at 37 °C and 5% CO₂ for 96 hours. Trypan blue dye was added to the wells at periodic intervals to determine the surviving cell counts using the hemocytometer. Cell survival was determined at 24, 48, 72 and 96 hours of incubation and survival percentage was calculated. Cell counts from the control samples, saline solution and genistein (200 $\mu\text{g/mL}$), were used for comparison.

Determination of the peptide mass and sequence using Mass Spectrometry

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used to identify the single peptides present in the fraction and to ascertain the approximate molecular size of the single peptide with highest activity. Serum albumin (70kDa) was used as a molecular standard. The peptide was separated from the gel matrix and sliced to small pieces before washing, to remove the stain, using 25mM ammonium bicarbonate solution in acetonitrile. The gel pieces with the peptide were washed in acetonitrile and dried before adding trypsin for digestion. The tryptic digests were analyzed for the sequences of amino acid residues using

liquid chromatography coupled with mass spectrometry (LC-MS) to separate the tryptic fractions and mass spectrometry (MS/MS) system to identify the amino acid sequences with the highest score. The samples were analyzed using spectrometer for emergence of peaks corresponding to single peptide based on their mass ratios (Grimm *et al.*, 1998).

The molecular mass of the peptide with highest activity identified and collected after chromatography studies was confirmed using the matrix assisted laser desorption ionization & time-of-flight (MALDI-TOF) Bruker Daltonics GMBH (Bremen, Germany) at the State Wide Mass Spectrometry Facility, University of Arkansas. All other HPLC grade chemicals were purchased from Sigma (St. Louis, MO).

Statistical analysis

All tests were conducted in triplicate to minimize experimental error and were repeated for reproducibility. The JMP 9.0 statistical software (SAS institute, Cary, NC) was used for all data analyses.

Results

Purification of the peptide fraction with highest activity to obtain single pure peptides

Based on the previous results (Rayaprolu *et al.*, 2013) from the cytotoxicity assay on the cancer cell lines, the 10-50 kDa peptide fraction with highest activity (70-73%) obtained from N98-4445A soybean line was selected for further purification. HPLC analysis of the 10-50kDa fractions revealed three peaks at varying elution times. The Figure 1 shows the isolation of three single peptides from the 10-50 kDa peptide fractions from N98-4445A soybean line. The semi-preparative HPLC was used with an affinity column to collect the peptides separately based on their elution times. The retention times for the three peptides were found to be 58, 67 and 79

minutes. The chromatogram identified the three peaks at distinct retention times. The three peptides were separately collected using the size exclusion column, concentrated, lyophilized and stored at 4 °C.

Anti-proliferative activity of pure peptides against blood, colon and liver cancer cell lines.

The single peptides collected from the chromatographic separation were tested for inhibition activity against the blood, colon and liver cancer cells. They were designated as E58, E67 and E79 based on their retention times during the chromatographic elution. The MTS assay determined the anticancer activity of the pure peptides, E58, E67 and E79, on the blood, colon and liver cancer cells (Figure 2). The test showed that the peptide E67 had significant anti-proliferative activity against colon and blood cancer cells with 74 and 80% inhibition respectively. The positive control, genistein, had higher comparatively inhibition of both the cancer cells. The E79 peptide showed highest activity of 75% inhibition against the liver cancer cells.

Although the peptide did not show a 100% kill on the colon cancer cells, it is significant to note its enhanced effect against cancer cell proliferation of 80% which can support its role in alternative cancer therapies. Previous studies have noted similar observation when a pure peptide, produced from rice bran and sequenced for amino acids, was tested for apoptotic molecular targets in MCF-7 breast cancer cells (Li *et al.*, 2014a). The pure peptides demonstrated significantly higher anti-proliferation activity on the three cancer cells in comparison to that of the peptide fractions (Rayaprolu *et al.*, 2013) from which they were purified, which was observed in our previous research. Based on the results the peptide E67 with the highest activity (80%) was further evaluated for cancer cell survival response using the Trypan blue dye exclusion assay to determine the impact of incubation time.

Time response study of the most effective peptide against human cancer cells.

The cell counts from the Trypan blue dye exclusion assay were used to quantify the effect of the peptide for reduction in cancer cell growth over time. The time response study showed that the peptide E67 reduced the survival of colon cancer cells to ~11% after 96 hours of incubation (Figure 3). However, the peptide reduced the CCRF-CEM blood cancer cell population by approximately 19% by the end of 96 hour incubation period. Similar observation was made by previous studies using single peptides that reduced cancer cell proliferation (Kannan, 2009). It was observed that the positive control genistein caused rapid cell death by 48 hour incubation and reduced the cancer cell population to almost 0% by 96 hours. It is also noted that the cell survival was reduced by >50% by 72 hours in both blood and colon cancer cells.

Determination of molecular size and characterization of the peptide with most significant anti-proliferation activity on human cancer cells.

The SDS-PAGE analysis showed the single peptides in the 10-50kDa fraction (Figure 7.4.). The approximate molecular mass of the most effective peptide ranged between 15-20kDa in the gel electrophoresis which was confirmed by the MALDI-TOF analysis to be 18kDa. The MS/MS spectra of peptides contained fragment ions, which result from single or multiple cleavages of peptide backbone bonds. The mass spectrometry (MALDI-TOF-MS) analysis located an intact peptide based on the single protonated molecular ions ($M+H^+$) which indicated the presence of the pure peptide that showed highest bioactivity in previous studies (Wysocki *et al.*, 2005, Tamvakopoulos, 2007, Murao *et al.*, 2007, Kannan *et al.*, 2010).

Characterizing a pure peptide and identifying its structural components confirms the peptide to its identity, and enhanced bioactivity. Absence of knowledge on the sequence of a peptide can explain the extraction of sequence information from MS/MS spectra or fragment ion

spectra through de novo sequencing. The tryptic hydrolysates with arginine or lysine residues at the N terminal when analyzed with MS revealed the peptide sequence which were matched with the soybean proteome for confirmation. The number of amino acids were identified from the C terminus end (Sequence) and a match with the National Center for Biotechnology Information (NCBI) database for the N-terminal amino acids showed a high prediction (>95%) with 158 amino acids in the peptide sequence. This peptide is recognized as the 2S albumin precursor from the soybean proteome provided in the NCBI database. The peptide sequencing with LC-MS and MS/MS analysis shown in Figure 5 (a, b and c) identified 158 amino acid residues in the sequence given as: MTKFTILLISLLFCIAHTCSASKWQHQQDSCRKQLQGVNLTPEKHIM EKIQGRGDDDDDDDDDNHILRTMRGRINYIRRNEGKDEDEEEEGHMQKCCTEMSELRS PKCQCKALQKIMENQSEEELEEKQKKKMEKELINLATMCRFGPMIQCDLSSDD-n.

Presence of multiple lysine, arginine and cysteine amino residues in the sequence potentially contributed to the anti-proliferation activity on cancer cells which is in congruence with previous research (Usui *et al.*, 2004, Cooper *et al.*, 2011, Nakase *et al.*, 2012). Medium sized (<50kDa) bioactive peptides as have been isolated and purified from various cereals and legumes (Korhonen and Pihlanto, 2003). Hence, this advocates and confirms the production of a cancer anti-proliferative peptide from soybean protein isolate after enzymatic hydrolysis.

Conclusion

Soybean is a good source to derive a pure bioactive peptide that showed 80% inhibition human colon and blood cancer cells in comparison to the 10-50kDa peptide fraction (73%) from which it was purified. Purification of the peptide fraction resulted in a potent single peptide with

highest anti-proliferative activity that can be tested against other prominent cancer cells. This research has potential implications in inexpensive and alternative cancer therapy.

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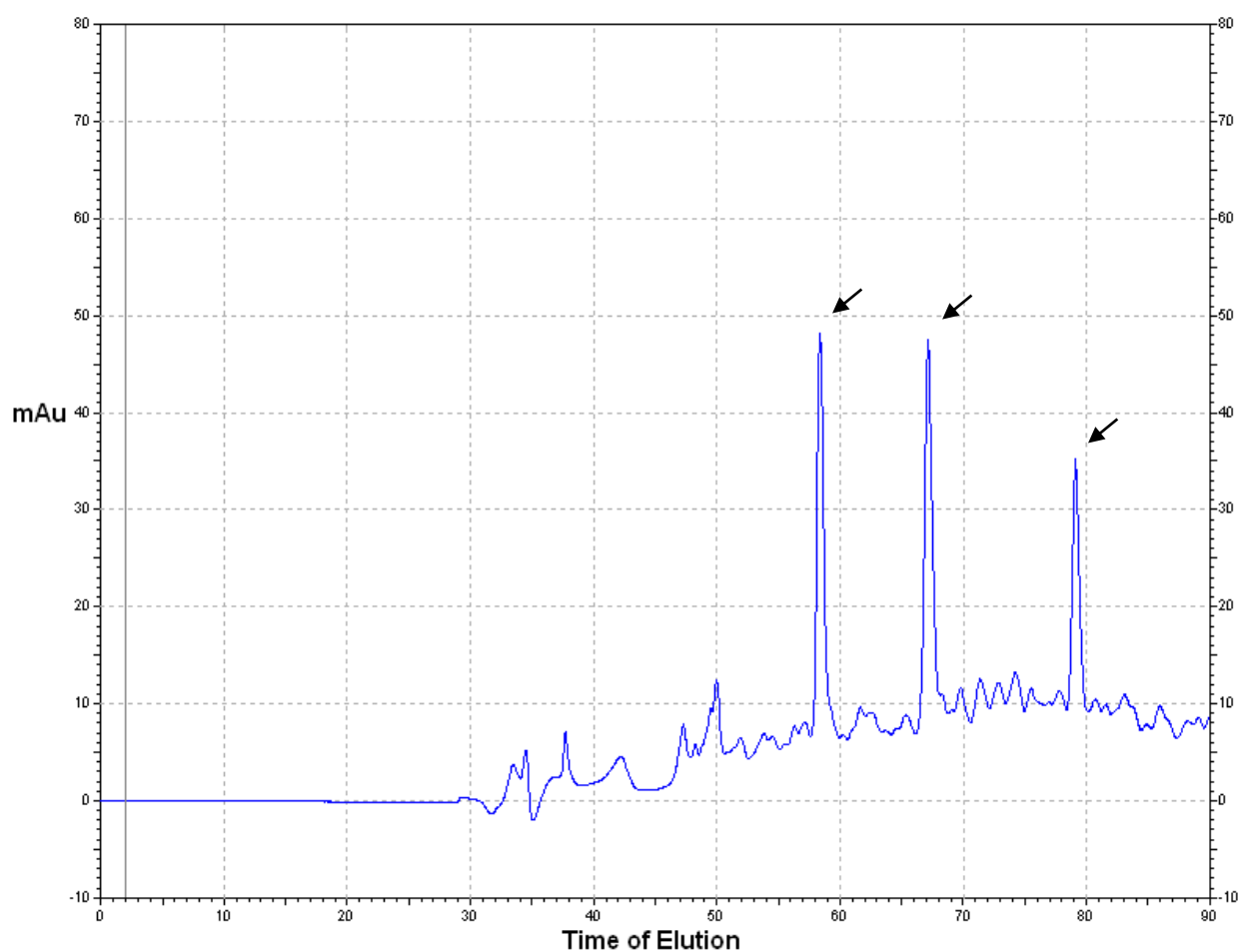


Figure 7.1. HPLC analysis of the 10-50 kDa peptide hydrollysate from N98-445A with three eluents identified based on time and peak area. Acetonitrile (50%) and water (100%) with Trifluoro Acetic acid (0.1%) was used as the solvent system to purify the peptides. Single peaks were depicted by the arrow heads.

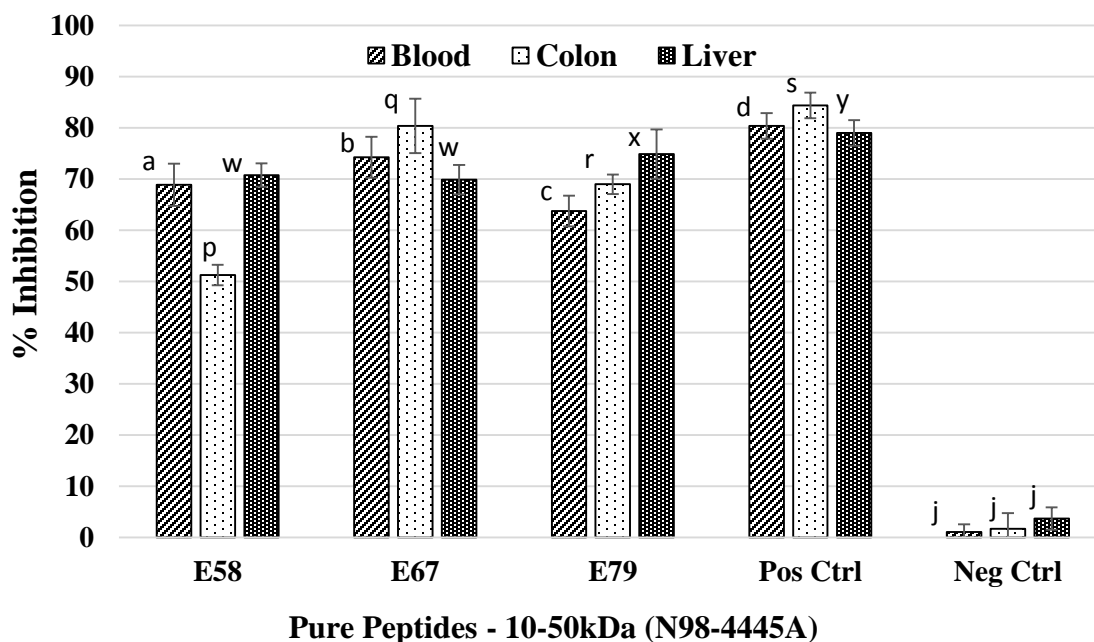


Figure 7.2. Single peptides purified from 10-50kDa fraction of N98-4445A soybean line show enhanced anti-proliferative activities against human blood, colon, and liver cancer cell lines. Bars represent means \pm standard deviations and those not connected with same letter are significantly different ($P < 0.05$). The dosage of the peptides applied was 700 $\mu\text{g/mL}$ while 1% saline solution was used as negative control (Neg Ctrl).

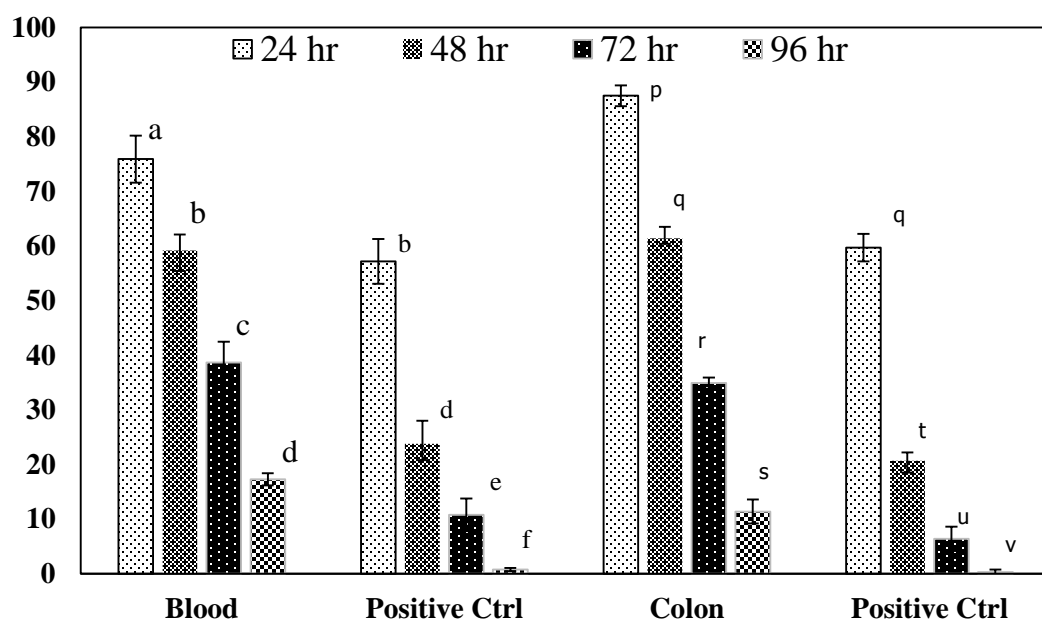


Figure 7.3. Confirmation of cancer cell survival reduction by the E67 peptide tested on HCT-116 colon cancer and CCRF-CEM blood cancer cells using Trypan blue dye exclusion assay. Bars presented are means \pm standard deviation and those not connected by same letter for each cancer type are significantly different ($P < 0.05$). Genistein (200 $\mu\text{g/mL}$) was used as positive control (Pos Ctrl) for comparison which was shown for each cancer type separately.

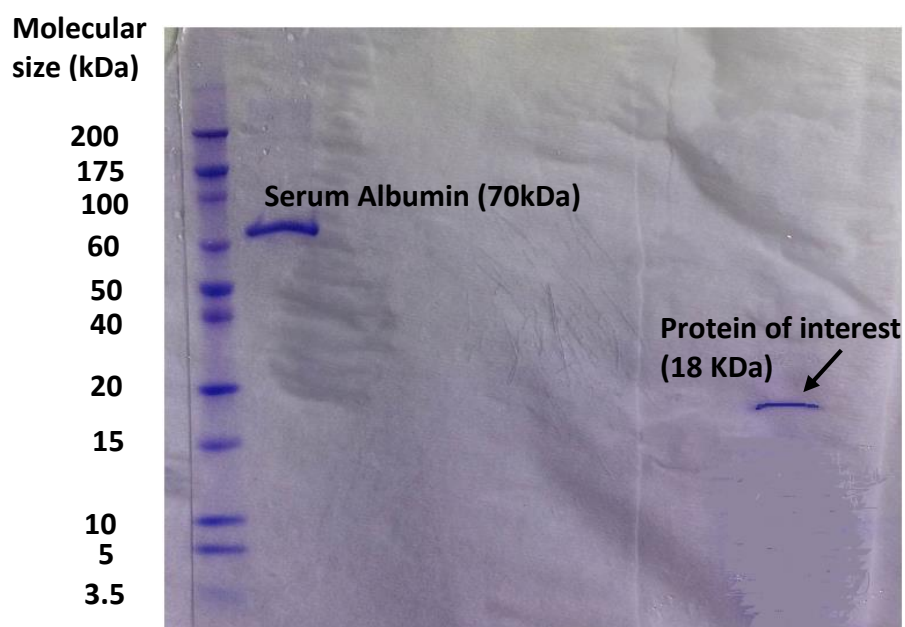
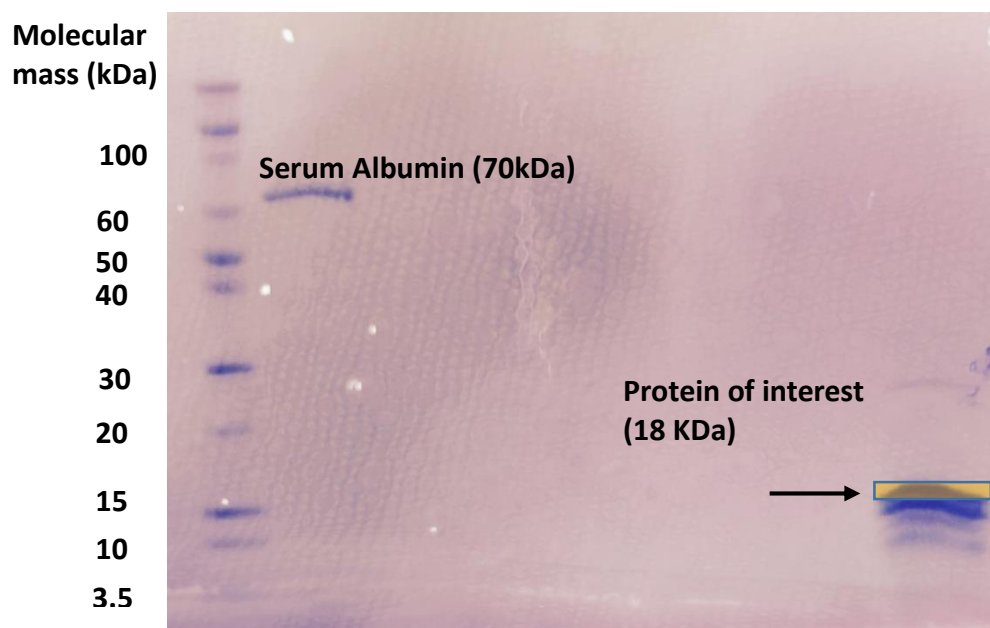


Figure 7.4 (a, b). Identification of the single pure peptide with significantly high activity against cancer cells using SDS-PAGE analysis. The arrow shows the anticancer peptide isolated from the N98-4445A soybean line. Serum albumin was used as a standard protein.

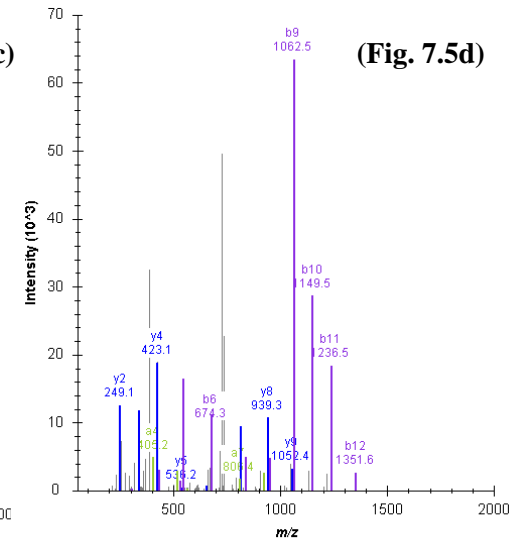
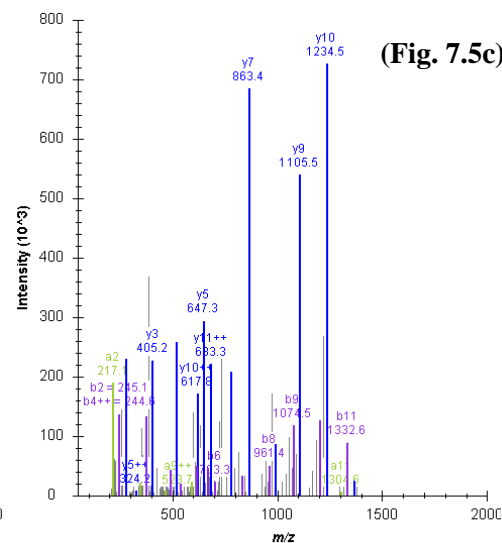
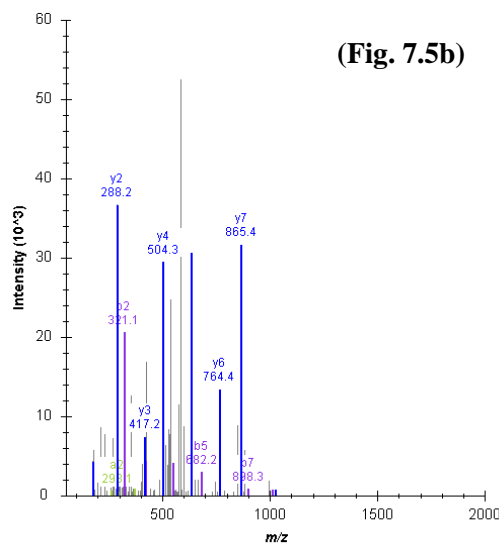
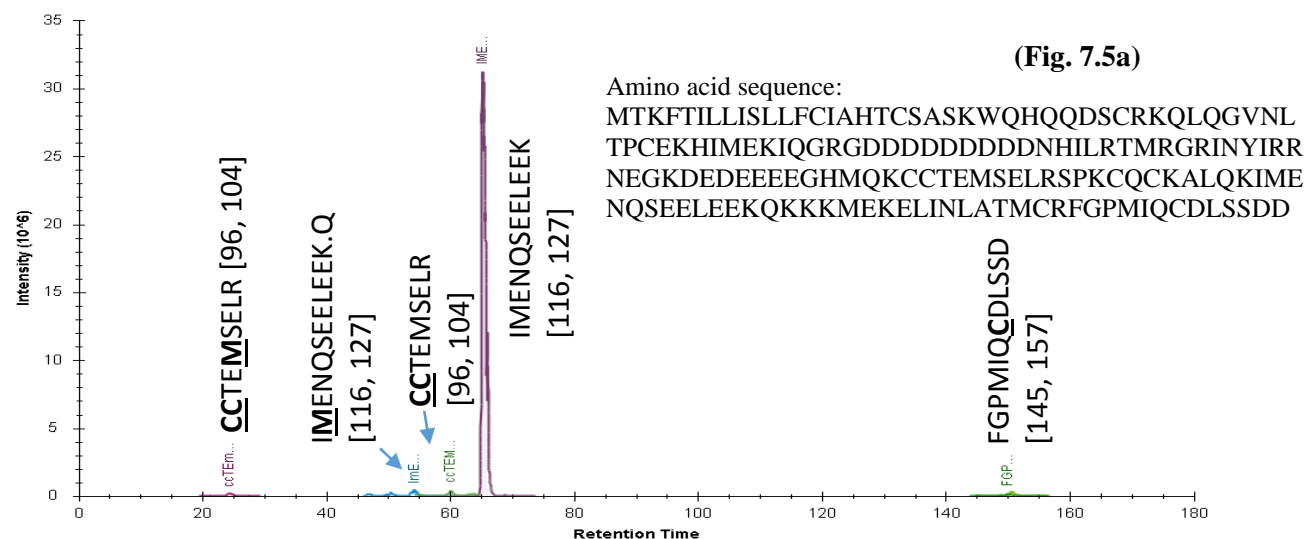


Figure 7.5 (a, b, c and d). Profile of the pure peptide sequence identified using LC-MS and MS/MS. Figure 7.5a shows the LC-MS analysis of the tryptic fractions in a sequence while the figures 7.5b - 7.5d show the MS/MS profiles of the three tryptic fractions which are used to identify the sequence of the peptide from the soybean proteome.

CONCLUSION

The basis for this research was utilization of an inexpensive co-product of soybean oil industry in developing novel gastrointestinal environment resistant biologically active peptides. These peptides showed effective inhibition of angiotensin-I converting enzyme (ACE-I), which regulates hypertension, and multiple-site cancer cell anti-proliferation activity. Higher protein content is a criterion for choosing soybeans as a good source for generating innovative bioactive peptides against chronic diseases. This is in congruence with FDA's recommendation that adding 25 g of soy protein in the diet can reduce risk of heart-related as well as other diseases. Currently soybeans with higher content ($\geq 85\%$) of oleic acid are becoming popular in the U. S. which can also be excellent source of proteins. This research has demonstrated that alkaline method of protein separation from the meal, hydrolysis using food-grade enzyme, evaluation of resistance to gastrointestinal (GI) juices and fractionation using ultrafiltration was favorable in deriving peptides that were GI resistant. Testing the potency of the GI resistant peptides by challenging them for activities including anti-ACE-I and multiple-site anti-proliferation of cancer cells is the characteristic aspect of this research.

Medium molecular weight 10-50kDa fractions and a pure peptide derived from the peptide pool demonstrated significant bioactive properties. This single peptide containing 158 amino acid residues in the sequence was characterized using mass spectrometry and established proteomic tools. This information on its structural components can be used for synthesizing the peptide for applications as a health promoting food ingredient, nutraceutical or for medicinal use. This can initiate future research in the direction of using protein products as components in alternative therapies for chronic human diseases.